THE ORAL SUSCEPTIBILITY OF SOUTH AFRICAN LIVESTOCK-ASSOCIATED *CULICOIDES* SPECIES TO SELECTED ORBIVIRUSES

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Thesis submitted in fulfilment of the requirements

for the degree of

PHILOSOPHIAE DOCTOR

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November 2007

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ACKNOWLEDGEMENTS

First, I want to thank René du Toit for showing that *Culicoides* midges are involved in the transmission of bluetongue and African horse sickness viruses, thereby opening the door for generations of scientists interested in the epidemiology of orbiviruses.

Research mentorship of Theuns van der Linde and Janusz Paweska is greatly appreciated. I am grateful to Janusz for his assistance, constant support and ongoing collaboration. Without his personal interest, unquenchable enthusiasm and sharing his virological expertise, this work would be difficult to accomplish timely. I am also thankful to his family Gosia, Magda and Piotr for lending him to *Culicoides* work for the last few years.

The contribution to my scientific development and continuous support of Professor Phillip Mellor from the Institute of Animal Health, Pirbright, UK is greatly valued, and especially for inviting me to be part of internationally funded projects on *Culicoides* vectors. I would like to thank Chris Hamblin and Simon Carpenter for collaborative studies.

A special thanks to Ina Hermanides, Dahpney Majatladi, Sandra Prinsloo, Isabel Wright, Solly Boikanyo and Karien Labuschagne for excellent laboratory and field technical support. I also thank Errol Nevill, Baltus Erasmus, Truuske Gerdes, Rudy Meiswinkel and Otto Koekemoer for advice and sharing their vast knowledge. I thank Ralph Burls from Clarens and the Onderstepoort Biological Products for making their animal holdings available for the collection of midges.

Vector competence studies were partly funded and supported by the European Union project “Development of a Safe, Efficacious Bluetongue Virus Vaccination Strategy for Europe” (Contract: QLK2-2001-01722), the National Department of Agriculture in South Africa (21.1.1/05AH-09/OVI) and the *Office International des Epizooties* Reference Centre for African Horse Sickness and Bluetongue at the Agricultural Research Council – Onderstepoort Veterinary Institute.

ABSTRACT

Culicoides (Diptera, Ceratopogonidae) midges play an essential role in transmitting orbiviruses. Recent outbreaks of bluetongue (BT) in Europe highlighted the risk for introduction and rapid spread of vector-borne diseases outside their traditional boundaries, and increase international interest in arbovirus epidemiology. African horse sickness virus (AHSV) and bluetongue virus (BTV) cause diseases of high socio-economic impact, especially on international trade. Identifying potential vectors is crucial for the implementation of integrated control measures, disease risk analysis and management. Determination of oral susceptibility of *Culicoides* species to infection with orbiviruses provides valuable data for assessing vector competence.

The aim of this work was to 1) determine oral susceptibility of livestock associated midges to infection with wild-type and live-attenuated strains of BTV and AHSV; 2) evaluate the efficiency of different light sources for the collection of *Culicoides* species; 3) compare laboratory blood-feeding methods; 4) identify potential vectors and 5) determine field infection prevalence in *Culicoides* species in the winter rainfall region of South Africa during an outbreak of AHS.

Although black light collection did not have any influence on the age-grading determination of a *Culicoides* population, it was more effective than white light for the collection of adult *Culicoides* midges. Cotton wool feeding yielded lower infection rates than membrane feeding due to the greater blood meal size taken by *Culicoides* females fed through the latter. Virus recovery of reference and vaccine strains of BTV was higher in *Culicoides bolitinos* than in *Culicoides imicola.* There was no significant difference between the oral susceptibility of *C. bolitinos* and *C. imicola* for the various AHSV isolates used. Virus recovery of the vaccine strain of AHSV serotype 7 (AHSV-7) from *C. imicola* at Onderstepoort was higher than that of the field strains of AHSV-7. *Culicoides imicola* from the eastern Free State

was more susceptible to AHSV than in Gauteng. Both BTV and AHSV were recovered from non-*Avaritia Culicoides* species. These results indicate BT and AHS to be multi vector diseases and add to the complexity of the epidemiology of orbiviruses. True assessment of vector competence might be difficult to assess if it would require some level of real-time monitoring, e.g. testing local *Culicoides* populations using variants of orbiviruses in current circulation.

Light trap surveys in the winter rainfall area of South Africa during an outbreak of AHSV demonstrated that *C. imicola* is superabundant and occurs in numbers to be equal or even higher than that in the summer rainfall areas. Results of oral susceptibility studies and the recovery of different orbiviruses, indicate this population of *C. imicola* to be highly vector competent.

KEY WORDS African horse sickness, artificial feeding, bluetongue, *Culicoides*, light trap collection, livestock, oral susceptibility, South Africa, vaccine

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Culicoides* biting midges

Culicoides biting midges (Diptera: Ceratopogonidae) are among the world's smallest haematophagous flies measuring from 1 to 3 mm in size (Mellor *et al.* 2000). The first reference to these insects is by Reverend W. Derham who described their life history and biting habits as early as 1731 (Mellor *et al.* 2000). The first research on sub-Saharan *Culicoides* dates to 1908 when two species were described from Namibia (Enderlein 1908). With the exception of Antarctica and New Zealand, *Culicoides* midges are found on virtually all large landmasses ranging from the tropics to the tundra (Mellor *et al.* 2000). Most *Culicoides* species possess unique grey and white-patterned wings which are useful for identification (Boorman 1993; Meiswinkel *et al.* 2004c). To date more than 1 400 *Culicoides* species belonging to 38 subgenera have been identified worldwide, of which at least 120 species are found in South Africa. They feed on a broad spectrum of hosts including reptiles, mammals, birds, man (Meiswinkel *et al.* 2004c), and even blood-engorged mosquitoes (Wirth & Hubert 1989). They are a severe biting nuisance to humans in certain parts of the world, cause an acute allergic dermatitis in horses, and are biological vectors of viruses, protozoa and filarial nematodes affecting birds, humans, and other animals (Linley 1985; Mellor *et al.* 2000; Meiswinkel *et al.* 2004c). Summer seasonal recurrent dermatitis, commonly referred to as sweet-itch, results from the bites of various species of *Culicoides* midges (Braverman 1988).

However, as vectors of viruses, *Culicoides* species are of the greatest medical and veterinary importance. More than 75 arboviruses, belonging mostly to *Bunyaviridae*, *Reoviridae* and *Rabdoviridae* families, were isolated from different

Culicoides species worldwide. Among viruses transmitted by *Culicoides* species, those causing bluetongue (BT), African horse sickness (AHS), equine encephalosis (EE), epizootic haemorrhagic disease of deer (EHD) and Akabane (AKA) disease are of major veterinary significance (Meiswinkel *et al.* 2004c). Bluetongue virus (BTV), in particular, has become of increased veterinary interest in the last decade due to its widespread in Europe since 1998 (Purse *et al.* 2005). The most important *Culicoides* vectors of orbiviruses include *Culicoides* (*Avaritia*) *imicola* Kieffer in Africa, *Culicoides* (*Monoculicoides*) *sonorensis* Wirth & Jones in North America, *Culicoides* (*Hoffmania*) *insignis* Lutz in South and Central America, *Culicoides* (*Avaritia*) *wadai* Kitaoka, *Culicoides* (*Avaritia*) *brevitarsis* Kieffer, *Culicoides* (*Avaritia*) *actoni* Smith in Australia, *Culicoides* (*Avaritia*) *fulvus* Sen & Das Gupta, *Culicoides* (*Remmia*) *schultzei* Enderlein in Asia, *C. imicola, Culicoides* (*Culicoides*) *pulicaris* L. and *C. imicola* and *Culicoides* (*Avaritia*) *obsoletus* Meigen in Europe (Mellor 2004; Tabachnik 2004).

1.2 Life-cycle

All *Culicoides* species display a typical holometabolous life-cycle and only the females, who need blood for the completion of the gonotrophic cycle, are haematophagous. No individuals are seen with partly developed eggs together with a fresh blood meal, nor with partly developed eggs without a partly digested blood meal, indicating normal gonotrophic harmony and a lack of autogeny in most South African species (Walker & Boreham 1976a). In *C. imicola* the maturation of eggs takes two to four days depending on the environmental temperature after a blood meal had been taken (Nevill 1967; 1969). The larvae undergo four stages, are eel-like in their movements, and burrow in and out of their breeding medium. The larvae of some species are carnivorous and feed on protozoa, rotifers and nematodes (Linley 1979). The fourth stage larvae of some species may even be cannibalistic on second

stage larvae (Nevill 1967; 1969). On immersion, the pupae of all species, except *C. imicola*, wriggle free of the breeding medium and float to the surface. *Culicoides imicola* pupae, however, lay on the substrate below the water surface and drown within two days at room temperature (Nevill 1967). It has been shown that soaking rains have no adverse effect on the eggs, larvae and pupae of most species, but the pupae of *C. imicola* do drown (Nevill 1967). The larvae of *C. imicola* will, however, not pupate until conditions are dry enough (Nevill 1967). Depending on the temperature adult *Culicoides* females may survive for up to 63 days (Nevill 1971). It is believed that all *Culicoides* species only breed in moist low-lying areas. Although this is true for some species, many have more specialized larval habitats (Nevill 1968; Dyce & Marshall 1989; Meiswinkel *et al.* 2004c; Nevill *et al.* 2007). The basic requirements are moisture and a medium containing organic matter. Therefore, *Culicoides* species may breed in situations varying from those which are almost aquatic, e.g. pond margins, to those where no free water is present but the humidity is close to 100%, e.g. interior of dung pads and decomposing fruit (Meiswinkel *et al.* 2004c).

1.3 Geographical and seasonal abundance of livestock-associated *Culicoides* species in South Africa

Over the last 35 years more than 112 *Culicoides* species were identified in South Africa (Meiswinkel *et al.* 2004c). In 1971 *C. imicola* was shown to be the most abundant livestock *Culicoides* species in the Onderstepoort area of South Africa (Nevill 1971). The results of subsequent studies showed *C. imicola* to be the most abundant livestock-associated *Culicoides* species in the summer rainfall area of South Africa, especially in the warm, frost-free summer rainfall areas of the country (Meiswinkel 1989; Venter 1991; Venter *et al.* 1996b). *Culicoides imicola* is relatively uncommon in warm/dry and cool/wet areas and therefore cannot be

regarded as the only vector of orbivirusses in South Africa (Venter 1991; Venter *et al* 1996b). The most abundant species in the latter areas were members of the *C. schultzei* group and *Culicoides* (*Hoffmania*) *zuluensis* de Meillon (Venter 1991; Venter *et al.* 1996b).

Some of the abundant and more widely distributed *Culicoides* species have a limited host preference and will thus be less important as potential vectors of orbivirusses (Nevill & Anderson 1972; Venter 1991; Nevill *et al.* 1992b; Venter *et al* 1996b*;* Meiswinkel *et al.* 2004c). According to these surveys, the more abundant and widespread species, which have the greatest potential as arbovirus vectors, are *C. imicola*, the *C. schultzei* group, *C. zuluensis*, *Culicoides* (*Beltranmyia*) *pycnostictus* Ingram & Macfie*, Culicoides* (*Meijerehelea*) *leucostictus* Kieffer*, Culicoides* (Unplaced) *bedfordi* Ingram & Macfie*, Culicoides* (*Culicoides*) *magnus* Colaço*, Culicoides* (Unplaced) *ravus* Das Gupta*, Culicoides* (*Avaritia*) *gulbenkiani* Caeiro*, Culicoides* (Unplaced) *similis* Carter, Ingram & Macfie and *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel (Venter 1991; Nevill *et al.* 1992b; Venter *et al.* 1996b).

A seven year study on the seasonal abundance of *C. imicola* at the ARC-Onderstepoort Veterinary Institute (ARC-OVI) showed a drop in adult numbers during sustained rainy periods followed by a sharp increase in populations during the drier periods that followed (Nevill 1971). A three year light trap survey indicated adults of *Culicoides* species, and especially *C. imicola,* to be present throughout the year in frost-free areas of the country and that breeding takes place throughout the winter in these areas (Venter 1991; Venter *et al.* 1997). In the major part of South Africa *Culicoides* numbers reach a peak in late summer and drop sharply after the first frost (Venter 1991; Venter *et al.* 1997). Low numbers of adult *Culicoides* midges during the winter months may not only be due to low temperatures but also to lower winter rainfall. Relatively large *Culicoides* collections can be made during

winter in the winter rainfall areas. No seasonal fluctuation of the dominant species in most summer rainfall areas was found (Venter 1991; Venter *et al.* 1997).

Culicoides imicola was absent in light trap collections made in the sheep farming area in the Karoo region of South Africa (Jupp *et al.* 1980) which is endemic for BT. This suggested that other livestock-associated *Culicoides* species may play a role in the epidemiology of this disease. *Culicoides imicola* is uncommon in the colder high-lying BT endemic areas of South Africa where *C. bolitinos* was found to be the most abundant *Culicoides* species (Venter & Meiswinkel 1994). *Culicoides bolitinos* was also shown to be abundant in the winter rainfall region of the Western Cape Province (Venter *et al.* 1996b, 1997; Nevill *et al.* 1988), and the dominant *Culicoides* species, in the absence of *C. imicola,* in the sandy dunefields adjoining Port Elizabeth in the Eastern Cape Province (Meiswinkel 1997). The absence of *C. imicola* at Port Elizabeth and in light trap collections made at Struisbaai and Alexanderbay on the southern and western coastline were attributed to the sandiness of the soil (Meiswinkel *et al.* 2004b). Limited records suggest that *C. bolitinos* is most probably also widespread in Africa but, unlike *C. imicola,* is not known to occur outside the Afrotropical Region (Meiswinkel 1989). Modelling studies seem to confirm the apparent lower abundance of *C. imicola* in the winter rainfall region of the South Western Cape Province and the cooler high-lying areas of the country (Baylis *et al.* 1998).

1.4 Livestock viruses associated with *Culicoides* species

Worldwide, more than 50 arbovirusses have been isolated from a variety of *Culicoides* species, including 20 bunyaviruses, 19 reoviruses, and 11 rhabdoviruses (Meiswinkel *et al* 2004c). Important livestock viruses transmitted by *Culicoides* species include African horse sickness virus (AHSV), bluetongue virus (BTV), epizootic hemorrhagic virus (EHDV), equine encephalosis virus (EEV), Akabane

virus (AKAV), bovine ephemeral fever virus (BEFV) and the Palyam viruses (Meiswinkel *et al* 2004c). At least two of these, AHSV and BTV, cause diseases of such international significance that they have been allocated Office International des Epizooties, the World Organisation for Animal Health (OIE) list status. Diseases listed by the OIE have the potential to spread rapidly from one country to another, cause high mortality and morbidity in susceptible animals, and affect international trade in livestock and livestock products. It was recently shown that a third virus on this list, vesicular stomatitis virus can be transmitted by *C. sonorensis* in the United States (Drolet *et al.* 2005).

Nyabira and Gweru are Palyam group viruses and have only been isolated in southern Africa and, like Akabane virus, are associated with abortions in cattle, goats and sheep (Swanepoel & Blackburn 1976; Whistler & Swanepoel 1988). Viruses of the Palyam serogroup have a particular association with cattle, and the numerous isolations made from *Culicoides* species suggest they are competent vectors for Palyam viruses in southern Africa (Whistler *et al.* 1989; Nevill *et al.* 1992a). Nyabira virus, for instance, replicates well in *C. imicola* and *C. zuluensis*, but transmission trials have proved inconclusive (Braverman & Swanepoel 1981).

1.4.1 Bluetongue

Bluetongue (BT) was first reported more than 125 years ago with the introduction of European breeds of sheep into southern Africa (Howell & Verwoerd 1971). The causative agent, BTV, is a double stranded RNA virus, within the genus *Orbivirus* of the family *Reoviridae* (Borden *et al.* 1971). Bluetongue virus exists as a number of serotypes of which 24 have been identified to date. This virus is thought to infect all known species of ruminants (Barnard 1997), but severe disease usually occurs only in certain breeds of sheep and some species of deer (Taylor 1986; McLachlan 1994). Neitz (1933) showed blesbok (*Damaliscus albifrons*) to be susceptible to

experimental infection with BTV and evidence of unapparent infections have subsequently been found in many other species (Hoff & Hoff 1976; Lage *et al*. 1996).

Clinical signs in certain breeds of sheep may include fever, depression, nasal discharge, excessive salivation, facial oedema, hyperanemia, and ulceration of the oral mucosa, coronitis, muscle weakness, secondary pneumonia, and death. African antelope do not develop clinical disease (Verwoerd & Erasmus 2004). Canine fatalities and abortions have been found to be associated with a vaccine contaminated with BTV (Akita *et al.* 1994; Wilbur *et al.* 1994). Subsequently, evidence of natural BT infection in a number of African carnivores has been obtained and it is surmised that such infections follows the ingestion of carcasses of infected ruminants (Alexander *et al.* 1994).

Until 1995 the global distribution of BTV lied approximately between latitudes 35°S and 40°N, although in parts of western North America it may extend up to almost 50°N (Dulac *et al.* 1989). Within these areas the virus has virtually a worldwide distribution, being found in North, Central, and South America, Africa, the Middle East, the Indian sub-continent, China, Southeast Asia, and Australia (Mellor 1990). Before 1995 BTV has also at times made incursions into Europe, although it had not been able to establish itself permanently on that continent (Mellor & Boorman 1995). Since 1998, however, BTV has expanded its northwards distribution causing outbreaks of the disease over 800km further north than previously recorded in southern Europe (Purse *et al.* 2005) and several authors postulate that climate change has recently resulted in *C. imicola* expanding its range northwards in Europe (Wittman *et al.* 2001; Purse *et al.* 2005). Since 1998 BTV serotype -2, -4, -9 and -16 occur annually in southern Europe, and in September 2006 outbreaks of BTV-8 have been confirmed as far north as the Netherlands, Belgium, Germany and northern France (Thiry *et al.* 2006).

Outbreaks of reported BT disease in South Africa ranged from 21 to 100 annually (Gerdes 2004). Twenty-two of the 24 four serotypes of BTV occur in South Africa and currently only types 20 and 21 are exotic to the country (Verwoerd & Erasmus 2004).

Monitoring for BTV infection in *Culicoides* midges trapped at various sites in South Africa over a period of six years (1979 to 1984) revealed that 14 to 18 different serotypes were encountered every season, albeit at markedly varying frequencies (Gerdes 2004). Usually, three to five serotypes were isolated predominantly and these were often responsible for more than 60% of the total number of isolates for that particular season. These dominant serotypes were largely replaced by others the following season, only to become dominant again three to four years later. Such serotypes obviously possess a high epidemic potential. In this category were BTV serotypes 1 to -6, -8, -11 and -24. A second group of BTV serotypes was present at much lower levels but was encountered every season. In this category were BTV-9, -10, -12, -13, -16 and -19. A third group represented by BTV-7, -15 and -18 appeared sporadically and probably has a low epidemic potential (Verwoerd & Erasmus 2004). Bluetongue virus serotype 17, thought to be exotic, was isolated for the first time in 1985 and then in 1986 and 2000 (Gerdes 2004).

1.4.2 African horse sickness

Similar to BTV, AHSV is a double stranded RNA virus, within the genus *Orbivirus* of the family *Reoviridae*, which causes an infectious, non-contagious, disease of equids. The virus exists as nine distinct serotypes (McIntosh 1958; Howell 1962), all of which are endemic in sub-Saharan Africa.

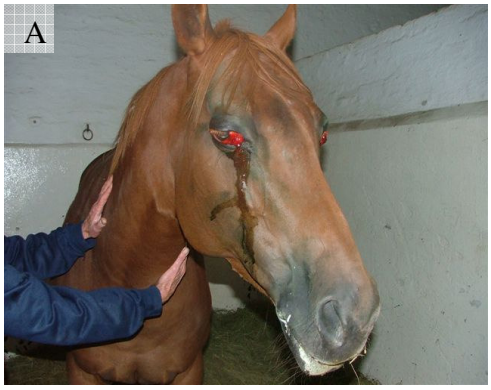
African horse sickness has been present in Africa for many centuries and in South Africa, where no indigenous horses existed, it was first noticed in 1652 after the introduction of horses from Europe and the Far East into the Cape of Good Hope

(Henning 1956). The disease was frequently mentioned in the records of the Dutch East India Company, and in 1719 nearly 1 700 horses succumbed to the dreaded 'perreziekte' or 'pardeziekte' in the Cape of Good Hope (Henning 1956). In 1854 - 55 a total of 64 850 horses succumb to AHS in the Cape Colony (Bayley 1856). The decline in the number of AHS outbreaks over the last few decades of the 20th century, particularly in the southern areas of South Africa, is partly due to the elimination of the large free-ranging populations of zebra (*Equus burchellii*), which are considered to be the natural cycling host for the virus (Barnard 1993; 1998; Mellor & Hamblin 2004). Of equal importance was the introduction of a polyvalent vaccine in 1974 which created a barrier of immune horses which apparently impedes the southerly spread of AHSV in South Africa (Bosman *et al*. 1995). Until 1990 the attenuated live-virus vaccine comprised two quadrivalent vaccines, one containing serotypes -1, -3, -4 and -5 and the other serotypes -2, -6, -7 and -8. Due to safety problems, the vaccine strain of AHSV-5 was discontinued in 1990 (Van Dijk 1998). Attenuation of these viruses has usually been achieved through three initial passages in mouse brain, followed by 10 passages in baby hamster kidney cell culture (BHK-21 cells) and, finally, by selection of avirulent large plaques in Vero cells (Erasmus 1976). The selected plaques were passaged further in Vero cells to prepare large volumes of master seed stocks. Working stocks for assembling polyvalent vaccines are currently produced in BHK-21 cells (Erasmus 1976; Paweska *et al.* 2003).

Traditionally AHS was found to be most prevalent in the northern parts of South Africa and major epidemics occur every 10 to 15 years. A strong link between the timing of these epidemics and the warm El Niño/Southern Oscillation (ENSO) has been demonstrated (Baylis *et al.* 1999). The endemnicity of AHSV in southern Africa greatly hampers the movement of horses from South Africa to Europe and the rest of the world.

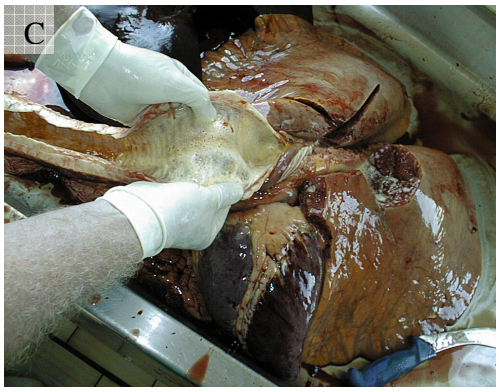
African horse sickness is characterized by clinical signs that develop from impaired function of the circularly and respiratory systems (Howell 1960; Coetzer & Erasmus 1994) and give rise to serous effusions and hemorrhage in various organs and tissues (Fig. 1). It can manifests in three ways, namely the lung form, the heart form and the mixed form. The lung form (dunkop) is characterized by a very high fever (>41 °C), difficulty in breathing, with mouth opened and head hanging down and a frothy discharge that may ooze from the nose. There is a sudden onset of death and the mortality rate is as high as 90%. The heart form (dikkop) is characterized by a fever, followed by swelling of the head and eyes and a loss of ability to swallow and possible colic symptoms. Terminal signs include hemorrhages in the membranes of the mouth, tongue and eyes. The onset is slower, death occurring 4 to 8 days after the fever had started. The mortality rate is about 50%. The mixed form is characterized by symptoms of both the dunkop and dikkop forms of the disease.

Periodically, AHSV expands out of sub-Saharan Africa and has caused major epizootics extending as far as Pakistan and India in the East, where more than 300 000 equids died during the great epizootic of 1959-61 (Howell 1960), and as far as Morocco, Spain and Portugal in the West (Mellor 1993). However, until recently the virus has not survived for more than two years in any of the epizootic areas. This short duration has been attributed to the absence of a vertebrate reservoir host in these regions of the world and to an absence or to a seasonal incidence of efficient vector species of *Culicoides.* However, the last outbreak of AHSV in the western Mediterranean basin, which lasted for five years (1987-91) has forced a reassessment of the situation (Mellor *et al.* 1990; Mellor 1993; 1994).

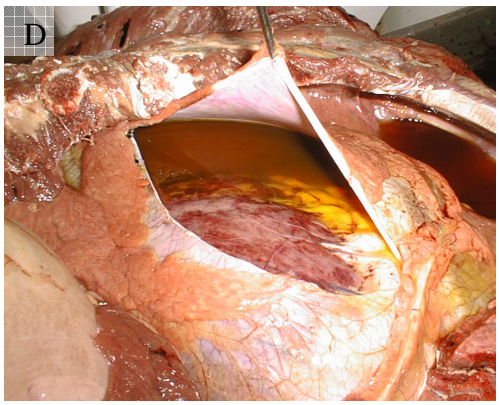


A - Severe conjunctivitis and swelling of supraorbital fossae

B - Discharge of froth from nostrils



C - Severe oedema of the interlobular septa in the lungs and froth in the trachea



D - Hydropericardium

Fig. 1 A-D Clinical signs and macroscopic pathology of African horse sickness (Courtesy of Dr J.T. Paweska)

1.5 *Culicoides* species as biological vectors of bluetongue and African horse sickness viruses

In 1943 Du Toit conducted the first successful transmission of BTV from infected *Culicoides* midges to susceptible sheep (Du Toit 1944). He also successfully infected a horse with AHSV by *Culicoides* bite (Wetzel *et al.* 1970). Currently it is accepted that both AHSV and BTV are transmitted between their hosts almost entirely by the bites of *Culicoides* midges. In consequence, distribution of these diseases is restricted to areas where competent vector species occur and transmission is limited to those times of the year when adult insects are active. In epizootic zones this usually occurs during the late summer and autumn, notably when outbreaks of AHS and BT are the highest (Mellor & Boorman 1995). The major *Culicoides* vector for BTV transmission differ in the broad geographic regions of the world, namely: *C. sonorensis* in North America, *C. insignis* in Central and South America, *C. imicola* in Africa and *C. wadai* and *C. brevitarsis* in Australia (Tabachnick 2004).

Of the 75 viruses isolated from *Culicoides* worldwide, 23 are from the Imicola Complex in the subgenus *Avaritia* Fox (Meiswinkel *et al.* 2004c). Currently the Imicola Complex comprises 13 species (Meiswinkel 1995). The most important orbivirus vector within this complex is *C. imicola*. It is the most widespread *Culicoides* species extending from the most southern tip of Africa northwards into southern Europe and eastwards as far as Laos, Vietnam and southern China (Meiswinkel 1989; Meiswinkel *et al.* 2004c). Based on it's abundance at livestock, it remains the most important vector of both BTV and AHSV in South Africa and the only confirmed field vector for these viruses (Nevill *et al.* 1992b; Meiswinkel *et al.* 2004c). Viruses isolated from field-collected *C. imicola* include AHS, BT, EE, Akabane, Bovine Ephemeral Fever (BEF), Nyabira, Sabo, Shamonda and Simbu (Nevill *et al.* 1992a; Halouzka & Hubálek 1996; Meiswinkel *et al.* 2004c).

In addition to *C. imicola,* other *Culicoides* species may act as competent field vectors of BTV in Europe (Mellor 1992; Mellor *et al.* 2000; Purse *et al.* 2005). It has been demonstrated that the expansion of BTV into areas of the Mediterranean Basin where *C. imicola* is rare or absent has been facilitated by at least two widespread and abundant Palearctic species groups, namely the *C. obsoletus* and *C. pulicaris* groups (Caracappa *et al.* 2003; De Liberato *et al.* 2003; Savini *et al.* 2005; Torina *et al.* 2004). In the Netherlands, also in the absence of *C. imicola, Culicoides* (*Avaritia*) *dewulfi* Goetghebuer was incriminated as a potential vector of BTV-8 (Meiswinkel *et al.* 2007).

Since *C. imicola* is rare or absent from some of the cooler or more arid areas where BTV is endemic (Jupp *et al.* 1980, Venter & Meiswinkel 1994; Meiswinkel *et al.* 2004c), it cannot be regarded as the sole vector of the virus. Recent studies have shown that, compared to *C. imicola, C. bolitinos* not only supported replication of BTV-1, -3 and -4 to higher titres (Venter *et al.* 1998) but also has a significantly higher transmission potential for BTV-1 over a range of different incubation periods and temperatures (Paweska *et al.* 2002). Furthermore, because this species is more common than *C. imicola* in many of the cooler BT enzootic areas and breeds in the dung of cattle (Nevill 1968), a major host species for the virus, *C. bolitinos* is likely to be the primary BTV vector in such areas (Venter & Meiswinkel 1994). In addition, non-*Avaritia Culicoides* species from South Africa such as *C. bedfordi*, *Culicoides* (Unplaced) *huambensis* Caeiro, *C. magnus, C. leucostictus, C. pycnostictus* and *Culicoides* (*Hoffmania*) *milnei* Austen were shown to be susceptible to oral infection with BTV-1 (Paweska *et al.* 2002). Similarly at least seven South African livestock-associated *Culicoides* species, belonging to at least six different subgenera, were shown to be susceptible to oral infection with attenuated strains of AHSV (Paweska *et al.* 2003).

1.6 Vector capacity and vector competence

The successful transmission of an arbovirus, from an infected to a susceptible host, is dependent upon the complex relationship that exists between the virus, its insect vector, the vertebrate host, and environmental conditions (Hardy *et al.* 1983).

Vectorial capacity refers to the ability of a vector population to transmit a pathogen. It can be defined as the average number of infective bites that will be delivered by a *Culicoides* midge feeding on a single host animal in one day and is a combination of midge density in relation to the host animal, host preference, midge biting frequency, life-span of infected midge, duration of viremia and vector competence (Dye 1992).

Vector competence is one of the factors which influences vectorial capacity and refers to the ability of a vector to support virus infection and replication and/or dissemination. It is a measure of the number of midges that actually become infective after feeding on a viraemic host and is dependent upon the genetic makeup of the vector midge and upon external environmental influences (Tabachnick 1991; Wellby *et al.* 1996; Mellor *et al.* 1998; Wittmann *et al.* 2001). Following ingestion by a susceptible arthropod, most arboviruses infect and replicate in cells of the mesenteron before penetrating the basal lamina to be released into the haemolymph to set up more cycles of infection and replication. As reviewed by Mellor *et al.* (2000) a number of barriers to arbovirus infection appear to exist, including mesenteronal infection and escape barriers, dissemination barriers, transovarial transmission barriers, and salivary gland infection and escape barriers (Fig. 2). In the North American *C. sonorensis,* the most important of these appeared to be the mesenteron infection barrier, which control initial establishment of persistent infection, the mesenteron escape barrier which can restrict virus to gut cells and the dissemination barrier which can prevent virus which enters the haemocoel from infecting secondary target organs (Fu *et al.* 1999). Although the expression of these

barriers appeared to be genetically controlled (Fu *et al*. 1999; Wellby *et al,* 1996), they can be bypassed by mechanical rapture of the midgut by e.g. filarial worms (Mellor & Boorman 1979) (Fig. 2). An arbovirus must first infect and replicate in the salivary glands before it can be transmitted during subsequent feeding on a susceptible host. The time from when the vector had ingested infected blood meal to excretion of the virus in the saliva is temperature dependent and takes one to two weeks (Fig. 2). In *C. sonorensis,* an apparent ovarian barrier excits which prevents transovarial transmission (Jones & Foster 1971; Nelson & Scrivani 1972; Nunamaker *et al.* 1990). However, recent studies demonstrated the presence of BTV nucleic acid by nested RT-PCR in *C. sonorensis* larvae (White *et al.* 2005) reinforcing the possibility of horizontal transmission of orbiviruses by *Culicoides* species.

A competent vector may have a low vectorial capacity due to low biting rates or survivorship, while a vector with low competence may be more efficient in virus transmission. For example, in Australia *C. brevitarsis* has a low competence for BTV, but effectively transmits the virus due to its high biting rate, while *C. fulvus* which is more competent, has a lower vectorial capacity due to lower abundance and limited geographical distribution (Standfast *et al.* 1985).

1.7 Experimental determination of vector competence

The virus vector competency of *Culicoides* species can be experimentally assessed by allowing midges to feed on a viraemic animal or on a blood virus suspension through a membrane in the laboratory. The engorged midges are then kept alive for the extrinsic incubation period i.e. the time from when the vector had ingested an infected blood meal until the excretion of the virus in the saliva. This takes usually one to two weeks (Fig. 2). The ability of infected midges to transmit viruses can then be assessed by allowing them to feed on susceptible animal hosts or alternative

substitutes, e.g. embryonated chicken eggs (Jones & Foster 1966; Foster & Jones 1973; Boorman *et al.* 1975).

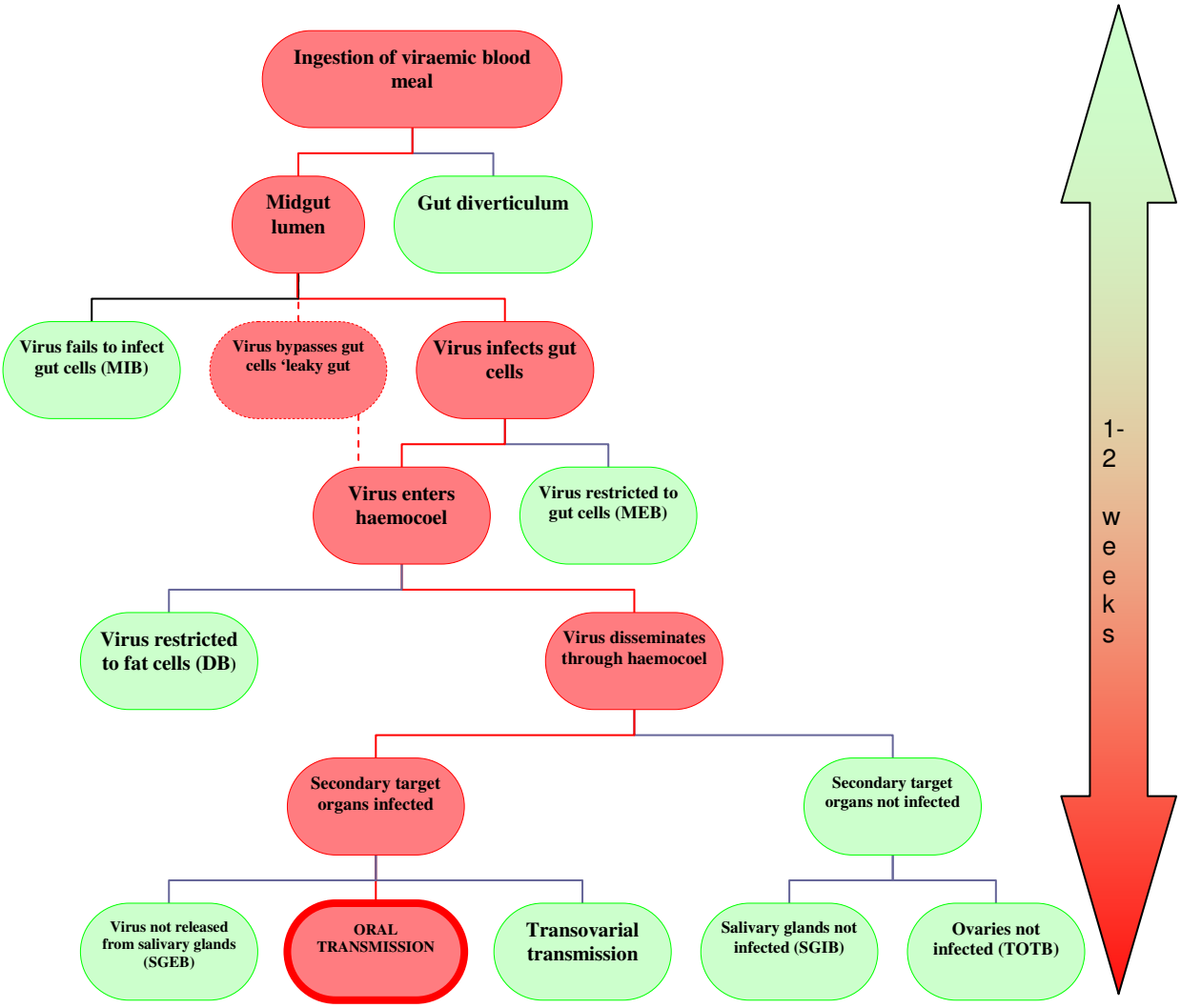


Fig. 2 Hypothesized barriers to arbovirus infection in haematophagous insects. (Adapted from Mellor *et al.* 2000). MIB = midgut infection barrier, MEB = midgut escape barrier, DB = dissemination barrier, SGEB = salivary gland escape barrier SGIB = salivary gland infection barrier and TOTB = transovarial transmission barrier.

1.8 Artificial infection methods

Methods for the artificial infection of *Culicoides* midges include the use of infected hosts (Foster *et al.* 1963; Luedke *et al.* 1967, 1976; Foster *et al.* 1968; Standfast *et al.* 1978; Muller 1985; Standfast *et al.* 1985; Jennings & Mellor 1988), embryonated

chicken eggs (Jones & Foster 1966; Foster & Jones 1973; Boorman *et al.* 1975), intrathoracic inoculation of the virus directly into the haemocoel of the midge (Boorman 1975; Mellor *et al.* 1975; Jennings & Boorman 1984; Muller 1987; Jennings & Mellor 1988), oral infection of *Culicoides* midges with virus using fine needles (Mellor *et al.* 1975), and feeding of *Culicoides* midges on cotton wool pledgets drenched with virus infected blood (Jupp *et al.* 1966; Carpenter *et al.* 2006) or membrane feeding methods (Jones & Potter 1972; Owens 1981).

Infected hosts are the most reliable method to use, but large numbers of *Culicoides* midges must be available at the time the infected host displays high viremia levels. Therefore, this method is only feasible when a *Culicoides* laboratory colony is available. The use of susceptible animals for transmission study with orbiviruses is expensive, time consuming and requires large laboratory space and insect proof stables. An alternative method is to use embryonated chicken eggs (Jones & Foster 1966; Foster & Jones 1973; Boorman *et al.* 1975). With a 30% infection rate in *C. sonorensis* with BTV it was possible to achieve 60% transmission rate when using embryonated chicken eggs (Jones & Foster 1966; Foster & Jones 1973). *Culicoides sonorensis* was also shown to transmit AHSV from infected to uninfected eggs (Boorman *et al.* 1975).

With intrathoracic inoculation the gut barrier is bypassed and species which are not susceptible after oral ingestion of the virus may become infected. Mellor *et al.* (1975) demonstrated that AHSV-9 replicates in both *Culicoides* (*Monoculicoides*) *nubeculosus* Meigen and *C. sonorensis* after intrathoracic inoculation but only in *C. sonorensis* after oral ingestion.

Cotton wool pledgets soaked with a blood/virus mixture are an easy and relatively inexpensive to use in large scale laboratory trails. A drawback of this method is that many arboviruses are cell-associated (Hoff & Trainer 1974) and the cells settle differently in a pledget. As result, the *Culicoides* females might be

feeding only on the serum dripping from the pledget. Therefore relatively high virus titres are required to successfully infect *Culicoides* midges.

Mellor & Boorman (1980) fed BTV to *C. nubeculosus* through a chick skin membrane and Jennings & Mellor (1987) determined the infection rate for colonized *C. sonorensis* through membrane feeding to vary from 0 to 51.6%. Jones & Potter (1972) used chicken skin membranes to design a six-position feeding apparatus for *C. sonorensis* and Owens (1981) successfully fed three Australian species of *Culicoides* on heated bovine blood through a stretched Parafilm membrane. Some European *Culicoides* species, however, showed an extreme reluctance to blood-feed through membrane based systems or upon live hosts under experimental conditions (Mellor *et al.* 1981; Jones *et al.* 1983; Mullen *et al.* 1985; Jennings & Mellor 1988; Mellor 1992; Goffredo *et al.* 2004; Carpenter *et al.* 2006).

1.9 Vector competence studies in South Africa

The oral susceptibility studies of Du Toit (1944) in South Africa, which for the first time clearly demonstrated the role of *Culicoides* midges in the transmission of orbiviruses, were subsequently confirmed elsewhere in the world i.e. in North America, Australia, and England, and then again in South Africa (Venter *et al.* 1991). In 1991 field-collected *C. imicola* was fed on sheep blood containing either BTV-3, -6 or AHSV-1. After an extrinsic incubation period of 10 days at 25-27°C, the rates of infections in *C. imicola* for BTV-3 and -6 were established at 31% and 24%, respectively. In this study, however, AHSV could not be recovered (Venter *et al.* 1991). In 1998 three serotypes of BTV were shown to have higher infection prevalence and higher virus titres/midge in *C. bolitinos* than in *C. imicola* (Venter *et al.* 1998). This important finding was confirmed by Paweska *et al.* (2002) who demonstrated that *C. bolitinos* had a significantly higher transmission potential for BTV-1 than *C. imicola* over a range of different incubation temperatures. Venter *et*

al (2004) first reported on the replication of four BTV attenuated strains in orally infected *C. imicola* and *C. bolitinos*.

Artificial feeding of 17 field-collected *Culicoides* species on blood containing three serotypes of AHSV resulted in infecting only *C. imicola* and *C. bolitinos* (Venter *et al.* 2000) but Paweska *et al.* (2003) reported recovery of live-attenuated vaccine strains of AHSV from experimentally infected six Old World livestock-associated non-*Avaritia Culicoides* species.

Of ten species fed artificially on the Bryanston serotype of EEV (EEV serotype 1), only *C. imicola*, *C. bolitinos* and *Culicoides* (Unplaced) *onderstepoortensis* Fiedler became infected (Venter *et al.* 1999, 2002). In a subsequent study 19 field-collected *Culicoides* species were fed artificially on each of six known serotypes of EEV. Of 19 *Culicoides* species assayed after 10 day extrinsic incubation, five yielded the challenge virus, namely *C. imicola* (EEV-1, -2, -3, -4, -5 and -6), *C. bolitinos* (EEV-1, -2, -4 and -6), *C. leucostictus* (EEV-1 and -2), *C. magnus* (EEV-1) and *C. zuluensis* (EEV-2) (Paweska & Venter 2004).

Artificial feeding of 17 *Culicoides* species on blood containing EHDV showed that eight of them were susceptible to oral infection with this virus (Paweska *et al.* 2005). Six of the eight EHDV serotypes were recovered from *C. imicola* and seven serotypes were recovered from *C. bolitinos.* Other *Culicoides* species that yielded EHDV after extrinsic incubation included *C. leucostictus C. magnus, Culicoides* (*Beltranmyia) nivosus* de Meillon, *C. gulbenkiani C. zuluensis* and *C. onderstepoortensis* (Paweska *et al.* 2005).

Of nearly 11 000 midges collected during two consecutive summers from two distinct climatic areas and fed on different strains of BEFV, all tested negative for the virus after 10 days extrinsic incubation. These results indicated that most of the abundant livestock-associated *Culicoides* species found in South Africa are refractory to oral infection with BEFV (Venter *et al.* 2003).

1.10 Study objectives

The primary aim of this study was to investigate the potential role that South African *Culicoides* species play in transmission of orbiviruses. In order to address this, the oral susceptibility of livestock-associated *Culicoides* species to various strains of BTV and AHSV were determined and isolations of orbiviruses from field-collected midges were attempted. Special attention was given to the comparison of vector competence of *C. imicola* and *C. bolitinos* which appear to be the major vector species for orbiviruses in South Africa. Large numbers of *Culicoides* midges are required for laboratory infection studies due to relatively high mortality in field-collected midges. Adequate collection methods are thus of paramount importance to ensure effective sampling. Alternative feeding methods as a mean of assessing vector competence have not been validated for South African *Culicoides* species. Specific objectives of this study were as follows:

1 Evaluation of black and white light sources for the sampling of South African livestock-associated *Culicoides* species.

2 Comparison of membrane and cotton wool pledgets as methods for artificial blood-feeding.

3 Susceptibility of *Culicoides* species to low-passaged reference and highly attenuated vaccines strains of BTV.

4 Susceptibility of *Culicoides* species to the vaccine strain and field isolates of AHSV-7.

5 Susceptibility of geographically distinct *Culicoides* populations to AHSV.

6 Identification of potential vectors and field infection prevalence in *Culicoides* species in the winter rainfall region of South Africa during outbreaks of AHS.

CHAPTER 2

EVALUATION OF BLACK AND WHITE LIGHT FOR THE SAMPLING OF SOUTH AFRICAN *CULICOIDES* SPECIES[1]

2.1 Introduction

In any arbovirus disease surveillance system and/or studies involving the use of live *Culicoides* midges, the principal aim is to capture the maximum number of vectors on near-by vertebrate hosts. In order to facilitate comparison of data and data sharing, standard techniques for measuring the variables of vectorial capacity should be developed and adopted. Currently the primary monitoring tools used for the capture of *Culicoides* midges are various models of white- or black light traps. Several factors may influence the number of *Culicoides* specimens as well as the number of each age grade collected with light traps (Venter *et al*. 1996a). These include the presence of breeding sites and other light sources near the light trap, the height of the trap above ground level, wind-speed, the phase of the moon, and even the tides. Climatic conditions such as temperature and wind velocity, rainfall, relative humidity, and the age of the population during the trapping night may also influence the numbers of *Culicoides* midges collected. It has long been realized that black light is more effective than white light for the collection of night-flying insects (Frost 1953; 1954), including *Culicoides* midges (Wieser-Schimpf *et al.* 1990; Rowley & Jorgensen 1967). Bishop *et al*. (2004) showed that light-emitting diodes, especially green and blue, were more efficient than a conventional light source in collecting Australian *Culicoides* species.

[1] Partially published as:

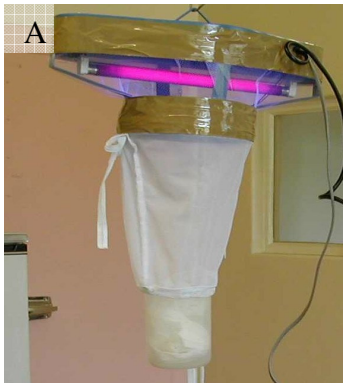
Venter, G.J. & Hermanides, K.G. (2006) Comparison of black and white light for collecting *Culicoides imicola* and other livestock-associated *Culicoides* species in South Africa, *Veterinary Parasitology*, **142**, 383-385.

Orbiviruses have been isolated so far only from parous individuals (Nelson & Scrivani 1972). There is no proof that orbiviruses can be transmitted transovarially by *Culicoides* midges (Jones & Foster 1971; Nunamaker *et al.* 1990). Therefore, the number of parous individuals in a population assayed is of paramount importance in determining their potential vector status. Black light was found to be preferable to incandescent white light for collecting parous *C. sonorensis* females (Anderson & Linhares 1989). The effectiveness of black and white light sources to attract and collect the different age stages of South African *Culicoides* species and especially *C. imicola,* is unknown. To address some of the above-mentioned issues, especially the numbers of *Culicoides* collected, species composition and parous rates, 220 V down-draught Onderstepoort light traps equipped with 8 W black and white light sources were compared under South African conditions.

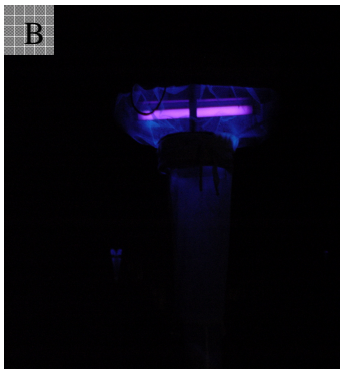
2.2 Materials and Methods

2.2.1 Light trap comparisons

Four 220 V down-draught suction light traps, two equipped with 8 W 23 cm black lights and two equipped with 8 W 23 cm white lights (Fig. 3), were used (Venter & Meiswinkel 1994; Goffredo & Meiswinkel 2004). Suction was provided by panel fans with an average air displacement capacity of 204.5 m^3/minute (STD = 9.47). These traps were deployed in two replicates of a 4 X 4 randomized Latin square design (Snedecor & Cochran 1980). The advantage of this design is that each treatment occurs once at each site and on each occasion. The treatment means are independent of any effects due to sites or occasion and, as only one treatment occupies a site on any occasion, trap interaction is avoided (Perry *et al.* 1980). Traps were operated from dusk to dawn under the eves of a stable, housing 15 to 20 horses at the ARC-OVI (25°39'S, 28°11'E; 1 219 m above sea level). Light traps were operated at opposite ends of the stable and were located 10 to 15 m apart.



A - Onderstepoort 220V down-draught black light trap



B – 23 cm 8 W black light tube

C - Midges collected into a 0.5% “Savlon” solution

Fig. 3 A-C Onderstepoort 220 V down-draught trap equipped with 23 cm 8 W black light tube

Trapping was conducted on ten consecutive nights in the early part of summer from 26 October to 4 November 2004. Due to adverse weather conditions no or very few *Culicoides* midges were collected on two nights and these treatments were repeated the following nights. Moths and other insects larger than 3 mm were excluded from the light trap collections by mosquito netting (apertures 2 mm) placed around the trap. Insects were collected directly into water containing 0.5% ‘Savlon’ antiseptic (contains Clorhexidine gluconate 0.3 g/100mL and Cetrimide 3.0 g/100mL) which breaks the surface tension and allows midges to drown and sink

to the bottom (Fig. 3C). After retrieval in the morning, collections were transferred to 80% ethanol and stored in the dark at 4 °C until counted and analysed as described in section 2.2.2.

2.2.2 Age-grading and data analyses

For age grading, the females of all species were classified according to the abdominal pigmentation method of Dyce (1969) into the following categories:

1 Nulliparous or unpigmented females

2 Parous or pigmented females

3 Gravid females with eggs visible in the abdomen

4 Freshly blood-fed females

5 To complete the analyses, males were also counted.

Data were analysed using the statistical program GenStat (2003). Log transformation was used to stabilise the variance.

2.3 Results and Discussion

A total of 69 027 *Culicoides* midges were collected in 32 light trap collections. The total numbers of each *Culicoides* species collected as well as the age-grading results are shown in Table 2.1. Significantly more (P<0.001, F=16.71, d.f.=1) *Culicoides* midges were collected with the 8 W black light source than with the 8 W white light source. With the black light source 51 041 *Culicoides* midges, belonging to 22 species were collected in 16 collections made (Table 2.1). The average number of midges collected per night with the black light source was 3 190.1 (STD=2 145.6).

With the white light source, 17 986 *Culicoides* midges belonging to 18 species were collected in the same number of collections made over the same period. The average number of midges collected per night with the white light source was 1 124.1 (STD=633.6) (Table 2.1).

Table 2.1 *Culicoides* species abundance and age structure as determined by 16 collections using black and white light. Trapping made near horses at ARC-OVI from 26 October to 4 November 2004

	N[1]		Nulliparous ♀		Parous ♀		Freshly Blood-fed ♀		Gravid ♀		Male		Total *Culicoides* (%)	
Light source	B[2]	W[3]	B	W	B	W	B	W	B	W	B	W	B	W
Culicoides species														
C. imicola	16	16	51.7[4]	53.9	42.4	40.4	0.4	0.6	2.2	1.2	3.4	4.0	48 894 (95.8)	16 976 (94.4)
C. bedfordi	16	16	33.0	19.1	15.0	4.4		1.5	25.8	32.8	26.3	42.2	388 (0.8)	204 (1.1)
C. leucostictus	16	16	16.1	15.4	7.8	5.6	0.2		43.7	38.5	32.2	40.6	435 (0.9)	143 (0.8)
C. pycnostictus	16	15	4.2	4.2	3.7	4.2		0.7	82.9	82.5	9.2	8.4	381 (0.8)	143 (0.8)
C. zuluensis	16	14	69.7	61.0	25.0	24.4			1.0	1.5	4.3	13.0	300 (0.6)	131 (0.7)
C. bolitinos	16	15	40.3	41.6	55.2	55.8		1.3	2.5	1.3	2.1		241 (0.5)	154 (0.9)
C. nevilli	15	16	52.8	66.7	30.8	25.0			1.1		15.4	8.3	91 (0.2)	84 (0.5)
C. magnus	14	14	53.3	52.9	43.3	44.3					3.3	2.9	90 (0.2)	70 (0.4)
C. nivosus	10	10	6.5	10.0		10.0			76.1	55.0	17.4	25.0	46 (0.1)	20 (0.1)
C. enderleini	8	3	49.1	33.3	26.4	50.0	5.7		3.8		15.1	16.7	53 (0.1)	6 (<0.1)
C. exspectator	8	9	2.6	7.1	2.6				94.7	78.6		14.3	38 (0.1)	14 (0.1)
C. similes	6	5	31.0	7.1	6.9	28.6			13.8	14.3	48.3	42.9	29 (0.1)	7 (<0.1)
C. neavei	5	4	11.1		33.3				16.7	50.0	38.9	50.0	18 (<0.1)	4 (<0.1)
C. trifascielus	3	8	33.3	33.3	33.3	58.3			33.3	8.3			6 (<0.1)	12 (<0.1)
C. nr glabripennis	4	5	37.5	33.3	62.5	33.3				33.3			8 (<0.1)	9 (0.1)
C. coarctatus	3	4	30.0	60.0	20.0	40.0			40.0		10.0		10 (<0.1)	5 (<0.1)
C. subschultzei	2	1		100							100		5 (<0.1)	1 (<0.1)
C. gulbenkiani	1	3		33.3						66.7	100		1 (<0.1)	3 (<0.1)
C. nigripennis	2		50.0								50.0		2 (<0.1)	
C. engubandei	1								100				2 (<0.1)	
C. brucei	2		100										2 (<0.1)	
C. schultzei	1								100				1 (<0.1)	
Total			50.8	52.7	41.4	39.1	0.4	0.6	3.5	2.7	4	4.9	51 041	17 986

[1]Number of collections in which the given *Culicoides* species was found, [2]220 V down-draught Onderstepoort light trap equipped with 23 cm 8 W black light tube, [3]220 V down-draught Onderstepoort light trap equipped with 23 cm 8 W white light tube, [4]Expressed as a percentage of the total catch

Despite the fact that four *Culicoides* species, *Culicoides* (Unplaced) *nigripennis* Carter, Ingram & Macfie, *Culicoides* (*Pontoculicoides*) *engubandei* de Meilon, *Culicoides* (*Culicoides*) *brucei* Austen and *C. schultzei* were collected in very

low numbers with black light (<0.1%), and not collected with white light, there was virtually no difference (r^2=0.98) in the *Culicoides* species composition sampled by the two light sources (Table 2.1).

Significantly more *C. imicola* (P<0.001; F=16.35, d.f.=1) were collected with the black light than with white light. *Culicoides imicola* was the dominant species in both the black light trap (95.8% of all *Culicoides* species collected) and white light trap (94.4% of all *Culicoides* species collected) (Table 2.1).

With traps equipped with a black light source, six species (*C. imicola, C. bedfordi*, *C. leucostictus*, *C. pycnostictus*, *C. zuluensis* and *C. bolitinos*) were present in each of the 16 collections made (Table 2.1). With the white light source, however, only four species (*C. imicola, C. bedfordi, C. leucostictus* and *Culicoides* (*Remmia) nevilli* Cornet & Brunhes) were present in each collection made (Table 2.1).

In general both trapping systems indicated that males, freshly blood-fed and gravid females, especially of *C. imicola*, were less attracted to light traps than parous and nulliparous females (Table 2.1). However, this was not true for *C. bedfordi*, *C. leucostictus* and *C. pycnostictus* where gravid females and males, singly or when combined, predominated in both light trap types. There was a very high correlation in the number of males, freshly blood-fed and gravid females (r^2=0.98 in all cases) of each species collected with the two different trapping systems. As expected significantly more (P=0.005, F=10.68, d.f.=1) insects other than *Culicoides* were collected with the black light source (average = 2 173.8) than with the white light source (average = 734.9). With both traps, however, the ratio of *Culicoides* midges to other insects was 1:5. Therefore, given the prerequisite that the light trap is covered with netting to exclude insects larger than *Culicoides* the use of a black light will not increase the time needed to sort *Culicoides* midges from these collections.

Black light was shown to be more effective in trapping *C. imicola* and other South African *Culicoides* species than white light. Since the most abundant *Culicoides*

species in an area might not represent the most competent vector species in transmitting a specific virus (Standfast *et al.* 1985; Venter *et al.* 1998; Paweska *et al.* 2002), the use of black light will not only increase monitoring sensitivity in areas where vector abundances are low but can be useful in the production of geographical risk maps and models for the various *Culicoides* species and associated disease transmission. Further benefits of using black light would be derived in areas and at times when *C. imicola* and other *Culicoides* vectors are not abundant, i.e. for first occurrence outside of endemic areas and at sites with low density and for detecting vectors at key locations involved in the export of livestock (staging areas and ports).

The number of parous individuals in a population is of paramount importance in determining the potential vector status of a *Culicoides* population (Nelson & Scrivani 1972; Nunamaker *et al.* 1990). Contrary to work done with *C. sonorensis* (Anderson & Linhares 1989), no significant difference was found in the parous rates for *C. imicola* as determined by traps equipped with black and white sources. In outbreak situations the prevalence of infection in populations of *Culicoides* vectors is very low (Meiswinkel & Paweska 2003; Savini *et al.* 2005). In this context, it is worthwhile to mention that White *et al*. (2005) detected BTV nucleic acid by nested RT-PCR in *C. sonorensis* larvae, suggesting that this virus may not require abundant expression of the outer coat genes to persist in the insect vector. This might explain the low rate of isolation of virus from insects and implies that transovarial transmission is possible. Black light traps will, however, provide more midges for virus isolation and thereby increase the possibility of identifying potential *Culicoides* vectors.

CHAPTER 3

EVALUATION OF ARTIFICIAL BLOOD-FEEDING METHODS[2]

3.1 Introduction

Since 1998 the Mediterranean Basin has been undergoing the most devastating incursion of BTV in recorded history, with severe economical losses (Mellor & Wittmann 2002; Calistri *et al.* 2003). During this incursion, novel Palaearctic *Culicoides* vectors, previously thought to be of limited epidemiological importance, have been implicated in the transmission of BTV in areas where the major European vector, *C. imicola* is absent (Mellor & Wittmann 2002).

Bluetongue virus has been isolated in the field from pools of field-collected specimens of two Palaearctic species complexes, the *C. obsoletus* group (Mellor & Pitzolis 1979; Savini *et al.* 2005) and the *C. pulicaris* group (Caracappa *et al.* 2003). However, laboratory competence studies of these complexes remain limited by an extreme reluctance on the part of field-collected individuals to blood-feed through membrane based systems or upon live hosts (Mellor *et al.* 1981; Jones *et al.* 1983; Mullen *et al.* 1985; Jennings & Mellor 1988; Mellor 1992; Goffredo *et al.* 2004; Carpenter *et al.* 2006).

Although feeding rates of up to 50% can be obtained in the *C. obsoletus* complex when they are offered cotton wool pledgets saturated with a blood/virus mixture following collection (Venter *et al.* 2005), this feeding method has not been validated as a mean of assessing vector competence.

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[2] Partially published as:

Venter, G.J., Paweska, J.T., Lunt, H., Mellor, P.S. & Carpenter, S. (2005) An alternative method of blood-feeding *Culicoides imicola* and other haematophagous *Culicoides* species for vector competence studies, *Veterinary Parasitology*, **131**, 331-335.

The aim of this study was to validate alternative means of feeding using the Afro-Asiatic BTV vectors *C. imicola* and the Ethiopian *C. bolitinos* as model species. They are relatively easily fed through membranes and via cotton wool pledgets, and are closely related to the *C. obsoletus* complex.

3.2 Materials and Methods

3.2.1 Infection rates of membrane and cotton wool pledget fed *Culicoides* midges

Culicoides imicola was collected in South Africa using down-draught, 220 V light-traps equipped with 8 W black light tubes from January to February 2000-2004 at the ARC-OVI, Onderstepoort (25°29'S, 28°11'E; 1 219m above sea level). Collections were also made during January to February 2000 on Koeberg Farm near Clarens (28°32'S, 28°25'E; 1 631 m above sea level) in South Africa where *C. bolitinos* predominates (Venter & Meiswinkel 1994; Meiswinkel & Paweska 2003). Handling of field-collected *Culicoides* before feeding was carried-out as described previously by Paweska *et al*. (2003). Bluetongue virus used in experiments were obtained from the OIE Reference Centre for Bluetongue at the ARC-OVI, South Africa (BTV-1 and -5), and from the Institute of Animal Health Pirbright (BTV-9) (Table 3.1).

Field-collected *Culicoides* were fed in batches of 300 to 500 for 30 to 45 minutes on defibrinated sheep blood spiked with one of three serotypes of BTV through a one-day-old chicken-skin membrane as described by Venter *et al.* (1991; 1998). Stocks of BTV serotypes were prepared as described by Paweska *et al*. (2003). In parallel, midges were also fed, immediately post-collection, on 2-3 cm^2 cotton wool pledgets saturated in blood/virus mixture (Jennings & Mellor 1988). Post-feeding *Culicoides* females were chilled and replete females separated and maintained in 250 mL unwaxed paper cups for 10 days at 23.5 ºC and 50-70% relative humidity. During incubation a 10% (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 µg streptomycin and 1.25 µg per mL of

fungizone) was made available via cotton wool pledegts. *Culicoides* surviving incubation were chilled and sorted into species before being stored individually in 1.5 mL microfuge tubes at -70ºC prior to virus isolation.

Table 3.1 Origin and passage history of bluetongue virus (BTV) isolates used in comparative oral susceptibility studies

BTV serotype	Origin and strain	Passage History
1	RSA 1958 (Biggarsberg)	50[1]E[2],3P[3],5BHK[4] (Howell 1969)
5	RSA 1953 (Mossop)	50E,2BHK,3P,7BHK (Howell 1969)
9	Kosovo 2001	2E,3BHK

[1]Number of passages, [2]Embryonated chicken eggs, [3]Plaque selection in green monkey kidney cells (Vero cells), [4]Production of virus stocks in baby hamster kidney cells (BHK-21 cells)

Isolation of virus in *Culicoides* females was carried in BHK-21 cell as described previously (Paweska *et al.* 2003). An antigen-capture ELISA (OIE 2004) and the virus neutralization test (Venter *et al.* 1998) were used for identification of virus isolates. Virus titres were calculated using the method of Kärber (1931). Fisher's exact test was used to compare the two methods of feeding and also to compare rates of virus isolation in *C. imicola* and *C. bolitinos*. A two-tailed Mann-Whitney test was used to compare the geometric mean virus titre in infected midges.

3.2.2 Comparison of blood meal sizes as determined by membrane and cotton wool pledget feeding methods

Feeding was carried out six times for membrane fed midges (pool size ranging from 300 to 500 midges) and five times for midges fed on cotton wool pledgets (pool size

ranging from 1 000 to 2 000 midges). The weights of the fed midges from each method were then compared to those of control groups of unfed midges. The blood meal size was calculated from the difference in weight, taking account of the specific gravity of the whole blood (1.06).

3.3 Results and Discussion

3.3.1 Infection rates in membrane and cotton wool pledget fed *Culicoides* midges

Infected individuals were recovered from both feeding methods, irrespective of the serotypes of virus used and species of *Culicoides* examined. In all cases, virus recovery rates (i.e. infection rates) were lower in midges fed via cotton wool pledgets than through membranes (Table 3.2). Although virus recovery rates were lower in insects fed via cotton wool pledgets than through membranes (Table 3.2), both methods yielded identical significant differences between *Culicoides* species and/or the BTV serotype involved. Higher infection rates in *C. bolitinos* than in *C. imicola,* for midges fed on BTV-1, were recorded with both membrane (P=0.003) and cotton wool pledget (P=0.001) feeding methods. Both methods showed that the infection rates of *C. imicola* fed with BTV-9 was significantly higher than those fed with BTV-1 (cotton wool pledget: P=0.001; membrane feeding: P<0.001). The infection rates of BTV-9 fed *C. imicola* were not significantly different to that for BTV-5 as determined by either method (cotton wool pledget: P=0.662; membrane feeding: P=0.055). The *C. imicola* infection rates for BTV-5 were significantly higher than with BTV-1 as determined using cotton wool pledget (P=0.009) or membrane feeding (P=0.001). Mean virus titre for midges (1.4 $\log_{10}TCID_{50}$/midge)[3] infected with BTV-1 (Table 3.2) did not vary significantly according to the method used to feed the two species (*C. imicola*: P=0.787; *C. bolitinos*: P=0.548), or between the

[3] TCID = Tissue culture infective dose

two species using the same feeding method (cotton wool pledget: $P>0.999$; membrane feeding: $P=0.383$).

Table 3.2 Virus isolation rates and titres in *Culicoides* midges fed using two different feeding methods and three serotypes of bluetongue virus (BTV)

BTV serotype		1			5			9	
Virus titre in blood meal ($Log_{10}/TCID_{50}$/mL of blood)		6.1			6.6			7.0	
Species	Cotton wool pledget	Membrane	P[3]	Cotton wool pledget	Membrane	P	Cotton wool pledget	Membrane	P
<u>*C. imicola*</u>									
Positive BTV isolations (%)	4/587[1] (0.7)	7/402 (1.7)	0.13[NS]	8/254 (3.1)	10/115 (8.7)	0.03[S]	14/337 (4.2)	77/480 (16.0)	<0.01[S]
Range in virus titres[2]	0.7-2.4	07-2.4	0.79[NS]						
Mean titre (STD)	1.4 (0.72)	1.4 (0.73)							
<u>*C. bolitinos*</u>									
Positive BTV isolations (%)	6/104 (5.8)	3/13 (23.1)	0.06[NS]						
Range in virus titres	0.7-2.4	1.1-2.4	0.55[NS]						
Mean titre (STD)	1.4 (0.58)	1.6 (0.70)							

[1]Number of individuals positive/ number of individuals tested, [2]$Log_{10}/TCID_{50}$/midge, [3]Comparison between feeding methods using Fisher's exact test, [NS]No significant difference, [S]Significant difference

3.3.2 Comparison of blood meal sizes as determined by membrane and cotton wool pledget feeding methods

The blood meal size calculated from pooled *C. imicola* fed via membranes ranged from 0.023 to 0.062 µl with a mean size of 0.045 µl in six trials. Using cotton wool pledgets the blood meal sizes ranged from 0.019 to 0.038 µl with a mean of 0.03 µl. Consequently, the mean blood meal size obtained with cotton wool pledget feeding was significantly smaller than when imbibing blood through a membrane ($P=0.048$).

Blood feeding *Culicoides* females using the cotton wool pledget feeding method produced infection rates that in comparison with membrane feeding methods and previously published data accurately reflected variations due to the serotype and titre of virus used. It also distinguished between a highly susceptible species (*C. bolitinos*) and a less susceptible one (*C. imicola*) (Venter *et al.* 1998; Paweska *et al.* 2002). However, the overall infection rates were significantly lower in cotton wool pledget fed midges. This was probably due, or at least in part, to a reduction in the size of the blood meal by about 30%. The size of the blood meal taken by *C. imicola* when using a membrane system was about 0.045 µl. This is not an absolute value as it does not take account of the elimination of excess liquid during feeding (Fujisaki *et al.* 1987; Leprince *et al.* 1989), but it can be used as a relative measure in comparisons between feeding methods. It also equates to the blood meal volume taken from the natural host by the similarly sized BTV vector *C. brevitarsis* (Muller *et al.* 1982).

Using the cotton wool pledget feeding method, Jennings and Mellor (1988) failed to record any positive results when attempting to infect the Palaearctic *C. obsoletus* complex midges with BTV. This discrepancy may be related to the fact that Jennings and Mellor added sucrose to the BTV/blood mixture which increases feeding rates but also resulted in diversion of most of the blood meal to the blind-ending acellular crop or mid-gut diverticulum (Mellor 1990; Venter & Carpenter, unpublished), which is not the normal route and may preclude virus establishing an infection. Alternatively, the population of *C. obsoletus* used by Jennings and Mellor (1988) had a very low level of oral susceptibility to BTV. In addition, these authors may have used insufficient numbers of midges to elicit an infection rate via the cotton wool pledget method. When using the pledget method in assessing the competence of potential vectors, it is important that a sufficient number of insects is used to compensate for a two to four fold reduction in infection rate as shown in the

present study. That criterion accepted, the cotton wool pledget method may prove useful when attempting to assess the competence of field-collected vectors that are reluctant to blood feed via a membrane or on the natural host.

CHAPTER 4

DETERMINATION OF ORAL SUSCEPTIBILITY IN SOUTH AFRICAN LIVESTOCK-ASSOCIATED *CULICOIDES* SPECIES

4.1 Introduction

Susceptibility of a vector insect to infection by an arbovirus, and the subsequent pattern of virus replication and dissemination in its tissues, is dependent on vector genotype and pathogen (Jones & Foster 1978; Jennings & Mellor 1987; Tabachnick 1991; Mecham & Nunamaker 1994; Fu *et al.* 1999; Wittmann 2000; Mullens *et al.* 2004; Mellor 2004) and on environmental factors (Mullens *et al.* 1995; Tabachnick 1996; Mellor *et al.* 1998; Wittmann 2000; Wittmann *et al.* 2002; Campbell *et al.* 2004). Laboratory demonstration of the potential of a particular species to support pathogen replication and transmission is one of the critical components aiding determination of vectorial capacity, which also depends upon biting rate, host selection, vector survivorship, and extrinsic incubation period of the virus (Mullens 1992; Mullens *et al.* 2004).

In this study the oral susceptibility of *C. imicola* and *C. bolitinos* and other livestock-associated *Culicoides* species are determined for various isolates and serotypes of BTV and AHSV. The virus isolates used originated from various geographical localities and include the reference and attenuated vaccine strains for these viruses.

4.1.1 Bluetongue virus reference strains[4]

In South Africa BTV has been isolated from field-collected *C. imicola*, *C. pycnostictus,* C*ulicoides exspectator* (Similis group) Clastrier (Nevill *et al.* 1992a; Meiswinkel *et al.* 2004c), and *C. bolitinos* (Meiswinkel & Paweska 2003). In addition, non-*Avaritia Culicoides* species from South Africa, namely *C. bedfordi*, *C. huambensis*, *C. magnus, C. leucostictus, C. pycnostictus* and *C. milnei* were shown to be susceptible to oral infection with BTV-1 (Paweska *et al.* 2002).

Recent vector competence studies indicated higher vector competence of *C. bolitinos* than *C. imicola* for BTV-1, -3 and -4 (Venter *et al.* 1998; Paweska *et al.* 2002). The aim of work presented in this section was to determine oral susceptibility of *C. imicola* to 13 low-passage reference serotypes of BTV in comparison to that of other livestock-associated *Culicoides* species, and especially *C. bolitinos*.

4.1.2 Bluetongue live-attenuated virus vaccine strains[5]

Concerns about the safety, including the development of viraemia in vaccinated animals and the potential for onward transmission by *Culicoides* midges in the field have most recently been addressed by Veronesi *et al*. (2005). *Culicoides sonorensis*, infected through feeding on vaccinated sheep, has been shown to transmit attenuated vaccine virus to susceptible animals (Foster *et al.* 1968). Reassortment between vaccine and wild type viruses may occur in the field and give rise to strains with novel virulence characteristics (Samal *et al.* 1987; Gibbs & Greiner 1988; El Hussein

[4] Partially published as:

Venter, G.J., Mellor, P.S. & Paweska, J.T. (2006) Oral susceptibility of South African stock associated *Culicoides* species to bluetongue virus. *Medical and Veterinary Entomology*, **20**, 329-334.

[5] Partially published as:

Venter, G.J., Mellor, P.S., Wright, I. & Paweska, J.T. (2007) Replication of live-attenuated vaccine strains of bluetongue virus in orally infected South African *Culicoides* species. *Medical and Veterinary Entomology,* **21**, 239-247.

et al. 1989; De Mattos *et el.* 1996; Kirkland & Hawkes 2004; Walton 1992). Therefore, data on oral susceptibility and vector competence of *Culicoides* midges for live-attenuated vaccine strains of orbiviruses are important for risk assessment analysis, particularly when intervention with live-attenuated vaccines is to be considered in regions vulnerable to virus incursions. Active circulation of vaccine BTV-2 among unvaccinated cattle in central Italy has been documented (Ferrari *et al*. 2005).

Adaptation of BTV to mammalian cell culture can result in altered characteristics of the virus (Gard 1987), including increased infectivity for vectors and changes in tissue tropism in the vertebrate hosts (Bellis *et al.* 1994). For example, it has been demonstrated that only tissue-culture attenuated strains of BTV are able to cross the ruminant placenta and cause teratological effects of the central nervous system of the foetus in sheep and cattle (Griner *et al.* 1964; Osburn *et al.* 1971; Dungu *et al.* 2004a; Verwoerd & Erasmus 2004).

Data on the vector competence of *Culicoides* midges for BTV strains that are included in the current commercial vaccines are very limited. The results of preliminary studies by Venter *et al.* (2004) indicated that the South African *Culicoides* species, *C. imicola* and *C. bolitinos,* are susceptible to oral infection with live-attenuated strains of BTV-1, -4, -9 and -16. The aim of this study was to determine the oral susceptibility of *C. imicola* and other livestock-associated *Culicoides* species to all cell culture-attenuated BTV vaccine strains that are currently used for vaccination of sheep in southern Africa. In addition, an inter-seasonal variation in oral susceptibility of C*ulicoides* species to infection with BTV was determined.

4.1.3 Wild-type and live-attenuated vaccine strains of African horse sickness virus serotype 7[6]

Similar to the attenuated strains of BTV, mouse brain (Riegler 2002) and tissue culture (Paweska *et al.* 2003) attenuated strains of AHSV were shown to orally infect *Culicoides* midges, which suggest that attenuation of this virus, either *in vivo* or *in vitro* systems does not necessarily affect its ability to infect *Culicoides* vectors. Paweska *et al.* (2003) demonstrated that *C. imicola* and *C. bolitinos* are susceptible to oral infection with most of the seven AHSV live-attenuated vaccine strains currently being used for massive annual vaccination of horses in South Africa, with the highest virus recovery rate reported for AHSV-7. Due to the lack of vector competence data for the non-attenuated parent vaccine strains, the question, to what extent attenuation process affects the ability of AHSV to infect *Culicoides* vectors, remains unknown.

With the aim of partially addressing this issue, the present study focused particularly on the comparison of the previously reported recovery rates in *C. imicola* and *C. bolitinos* for the AHSV-7 vaccine strain with that for field isolates of AHSV-7 recovered in 1998-1999 from naturally infected horses in different geographical regions of South Africa.

4.1.4 *Culicoides* population susceptibility to infection with African horse sickness virus

Vector species and different populations within a species may vary greatly in respect to susceptibility to BTV infection (Jones & Foster 1978; Ward 1994; Carpenter *et al.* 2006). Some field populations of a vector species may even be completely resistant

[6] Partially published as:

Venter, G.J. & Paweska, J.T. (2007) Virus recovery rates for wild-type and live-attenuated vaccine strains of African horse sickness virus serotype 7 in orally infected South African *Culicoides* species. *Medical and Veterinary Entomology*, **21**, 377-383.

to infection by some serotypes (Jones & Foster 1978; Ward 1994; Carpenter *et al.* 2006). In the United States, it was shown that the average infection rate of *C. variipennis* (= *C. sonorensis*) populations from a given state and seroprevalence of BT antibody in cattle from that state is strongly correlated (Tabachnick & Holbrook 1992). In South Africa, comparison of the oral susceptibility of *C. imicola* to geographical different isolates of AHSV-7 indicated significant differences for AHSV-7 isolates originating from different geographical localities. The highest virus recovery rate from the Onderstepoort population of *C. imicola* of an AHSV-7 strain isolated from a naturally infected horse at Onderstepoort might be indicative of a higher fitness between the virus and *Culicoides* vectors from the same geographical locality.

Unexpected large populations of *C. imicola* encountered at Clarens in the eastern Free State during the 2005/6 summer season made it possible to determine and compare the oral susceptibility of *C. imicola* populations from Clarens with that of *C. imicola* populations from Onderstepoort and *C. bolitinos* populations at Clarens.

4.2 Materials and Methods

4.2.1 Viruses

Reference, field-isolates, and live-attenuated vaccine strains of BTV and AHSV were obtained from the OIE Reference Centre for Bluetongue and Africa Horse Sickness, at the ARC-OVI. The strain identification, origin and passage history of the attenuated vaccine strains of BTV used are listed in Table 4.1. Stocks of vaccine and reference viruses for oral infection studies were grown in BHK-21 cells, titrated and stored in 10% foetal bovine serum in 1mL aliquots at -70 °C. Aliquots of virus stocks for oral infection were titrated during the experiments as described by Venter *et al.* (1998).

Table 4.1 Identification, origin and passage history of bluetongue virus (BTV) live-attenuated vaccine strains (Erasmus, B.J. 2006, Deltamune, personal communication)

BTV serotype	Strain identification	Origin	Passage history
1	Biggarsberg/8012	RSA, 1958	50E[1] 3P[2] 4BHK[3]
2	Vryheid/5036	RSA, 1958	50E 3P 4BHK
3	Cyprus/8231	Cyprus, 1944	45E 2BHK 3P 5BHK
4	Theiler/79043	RSA, ~1900	60E 3P 9BHK
5	Mossop/4868	RSA, 1953	50E 2BHK 3P 6BHK
6	Strathene/5011	RSA, 1958	60E 3P 7BHK
7	Utrecht/1504	RSA, 1955	60E 3P 7BHK
8	Camp/8438	RSA, 1937	70E 3BHK 10P 7BHK
9	University Farm/2766	RSA, 1942	50E 2BHK 3BHK 7P
10	Portugal/2627	Portugal, 1956	81E 6BHK
11	Nelspoort/4575	RSA, 1944	35E 3P 5BHK
12	Estansia/75005	RSA, 1941	55E 3P 4BHK
13	Westlands/7238	RSA, 1959	45E 2BHK 3P 4BHK
14	Kolwani/89/59	RSA, 1959	60E 3P 5BHK
16	Pakistan/7766	Pakistan	37E 3P 2BHK 1Vero[4]
19	143/76	RSA, 1976	29E 3P 3BHK

[1]Number of passages in eggs, [2]Number plaque selections, [3]Number of passages in baby hamster kidney cells, [4]Number of passages in green monkey kidney cells

To determine if passage of the live-attenuated vaccine viruses through an insect vector will alter their ability to infect vectors, field-collected midges were fed on blood spiked with midge isolates of the vaccine strains. Due to the relatively low virus recovery rates of BTV from orally infected *C. imicola*, these midge isolates were obtained from field-collected *C. imicola* females that had been intrathoracically inoculated with one or another of the vaccine strains of BTV-1, -2, -4, -9 and -16. Of 2 892 field-collected *C. imicola,* which were inoculated with the vaccine strains of BTV, 911 (31.5%) survived the 10 day incubation period at 23.5 °C. After incubation, surviving *C. imicola* females were pooled (*ca* 180 individual females/BTV serotype) and these pools titrated in BHK-21 cells to confirm infection and titre of virus present per pool. As the titre of virus obtained in this way was

invariably too low to use in vector oral infection studies, the virus was further replicated by passaging in *Ae. albopictus* C6/36 cells until the titre reached at least 7.0 $\log_{10}TCID_{50}$/mL of blood. To achieve virus titres between 5.8 and 6.8 $\log_{10}TCID_{50}$/mL of blood in the blood, the various midge isolates were passaged two to four times on *Ae. albopictus* C6/36 cell cultures. The oral susceptibility of field-collected *C. imicola* for these "vector passaged viruses" was compared to that of the same vaccine strains of the virus, but not passaged through the vector.

The identification and geographical origins of the AHSV-7 field isolates used are listed in Table 4.2. The geographical distances between the sites varied from 20 to 500 km. Field isolates of AHSV-7 were recovered from routine 1998 and 1999 diagnostic submissions to the OIE Reference Centre for African horse sickness. Viruses were isolated by standard inoculation of equine blood or homogenized organ samples into suckling mouse brains, followed by one or two passages in Vero cells. Serotyping was done by virus neutralization assays (House *et al.* 1990) using type-specific antisera produced in guinea pigs (Katz *et al.* 1993). Stocks of field isolates for oral infection studies were grown, titrated and stored as described by Paweska *et al.* 2003.

Table 4.2 Designation and origin of African horse sickness virus serotype 7 (AHSV-7) field isolates used for comparative oral susceptibility

AHSV-7 isolate designation	Origin (Province)	Year of isolation
23/98	Pietermaritzburg (Kwazulu Natal)	1998
1/99	Krugerdorp (Gauteng)	1999
3/99	Derdepoort (Gauteng)	1999
7/99	Onderstepoort (Gauteng)	1999
29/99	Rustenburg (North West)	1999

To compare the oral susceptibility of various geographical *Culicoides* populations to infection with AHSV, field-collected midges were fed with the

historical reference strain of the virus as well as with its recent field isolates. The geographical origin of the field isolates of AHSV-7 used is shown in Table 4.3.

Table 4.3 Designation and origin of the African horse sickness virus (AHSV) field isolates used for *Culicoides* population comparative oral susceptibility study

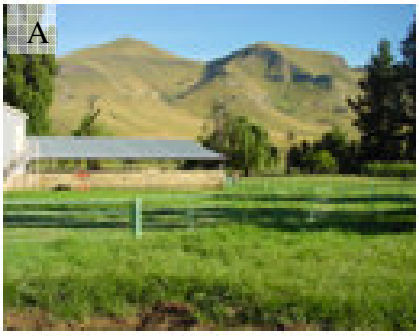
AHSV serotype (isolate designation)	Origin (Province)	Year of isolation
2 (4.04)	Grahamstowns (Eastern Cape)	2004
7 (2/04)	Lephale (Limpopo)	2004
9 (32/04)	Mokopane (Limpopo)	2004

4.2.2 Sampling of *Culicoides* midges for oral infection studies

Culicoides collections were done annually in the summer months (November to April) from 2002 to 2006. Adults of *C. bolitinos* were collected on Koeberg Farm near Clarens (28°32'S, 28°025'E; 1 631 m above sea level) in the high-lying eastern Free State. Light-trap collections were made near 300 sheep in an open-sided stable and near cattle in an open paddock (Fig. 4A). Eight to ten down-draught 220 V light-traps equipped with 8 W black light tubes (Fig. 3B) were used to collect live *Culicoides* midges into 500 mL plastic beakers (Fig. 4B-D). Midge survival was facilitated by excluding large insects from the collections using mosquito netting placed around the trap and a piece of roughly folded paper towel placed into the beaker to provide shelter for the trapped midges against the down-draught suction fan (Fig. 4C). Each morning catches were transfered into black boxes (450 mm x 330 mm x 280 mm) and sealed with ventilated lids (Fig. 5A,B). A white translucent exit funnel was mounted over a hole on one side of the box, onto which a 300 mL unwaxed paper cup was fixed (Fig. 5B). Active *Culicoides* in the black boxes move towards the light through the funnel into the collecting cup (Fig. 5B). These midges were kept in the dark and fed a 10% (w/v) sucrose solution containing antibiotics

(500 IU penicillin, 500 µg streptomycin and 1.25 µg per mL of fungizone) on cotton wool (Fig. 5C). The cups with *Culicoides* were then placed in insulated boxes and kept cool using ice packs for the duration of the five hour journey to the laboratory (Fig. 5C).

Adults of *C. imicola* were collected at 4 to 5 sites at the ARC-OVI (25°39'S, 28°11'E; 1 219 m above sea level). Animals near-by the light-traps were mostly cattle and horses. This site was chosen because of the very high numbers of *C. imicola* consistently found in this area (Venter *et al*. 1996a,b; 1997).



A - Sheep shed at Koeberg farm, Clarens, eastern Free Sate

B - One of ten black light traps in operation at Koeberg

C - Collection of live midges for oral susceptibility studies

D - Trapped live midge

Fig. 4 A-D The collection of live midges for oral susceptibility studies

A - Emergence box

B - Sorting of live *Culicoides* midges

C – Transportation of live *Culicoides* midges to Onderstepoort laboratory

Fig 5 A-C Collection and transportation of live midges for oral susceptibility studies

4.2.3 *Culicoides* feeding

Before feeding, the field-collected *Culicoides* were held without access to nutrients or water for 24 hours at 23.5 ºC and a relative humidity of 50-70%. Surviving flies were subsequently fed in batches of 250 to 300 for 30 to 40 minutes on defibrinated sheep blood containing a high titre of one of the virus isolates. Feeding was through a one-day-old chicken-skin using a technique described by Venter *et al.* (1998) (Fig. 6-8). Bluetongue virus and AHSV in the blood is primarily associated with erythrocytes and, to a lesser extent, with the buffy coat fraction (Hoff & Trainer 1974). To prevent midges from feeding on the plasma instead of the red blood cells some of the plasma was removed before adding the stock virus. During the feeding

period, illumination in the room was dimmed to ~1% daylight (~65 lux). A blood-virus mixture (Fig. 7C) was freshly prepared immediately before feeding. The blood-virus mixture was maintained at 35.5 ºC and stirred slowly during feeding (Fig. 7D). Blood-engorged females were separated out on a refrigerated chill-table and incubated for 10 days at 23.5 °C (Venter *et al.* 1998) (Fig. 8D). Engorged females (Fig. 8B) were maintained on 5% (w/v) sucrose solution containing 500 IU penicillin, 500 µg streptomycin and 1.25 µg fungizone per mL of sucrose solution. All *Culicoides* females surviving the incubation period were sorted to species on a chill-table (Fig. 8A-C) and stored individually in 1.5 mL microfuge tubes at -70 ºC until assayed.

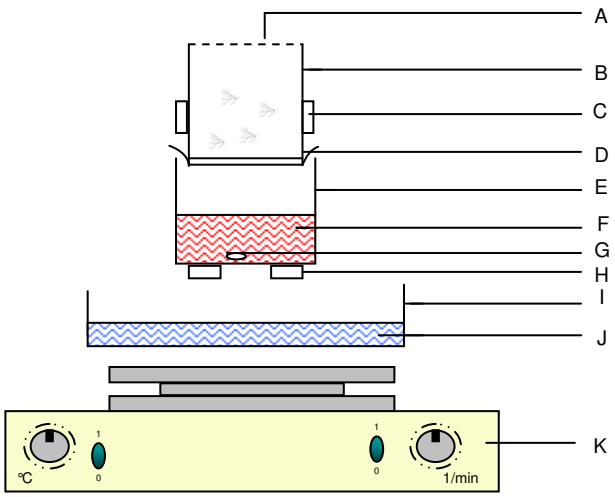


Fig. 6 Blood-feeding device for field-collected *Culicoides* species: A = gauze top; B = feeding chamber (40 mm diameter plastic "pill-bottle"); C = foam rubber; D = one day old chicken skin membrane; E = blood container (45 mm diameter plastic "pill-bottle"); F = blood/virus mixture kept at ±37°C; G = magnetic stirrer bar; H = rubber stopper support; I = water-bath; J = water; K = magnetic heater/stirrer (redrawn from Venter *et al.* 1991)

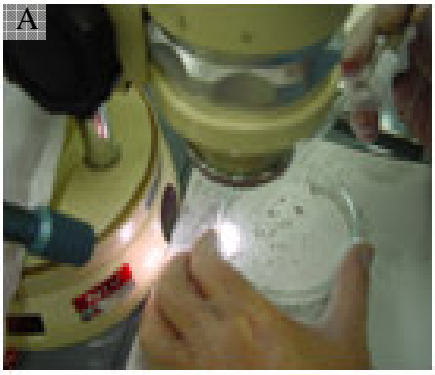
A - Blood container with magnetic stirrer bar

B – Midges in feeding container

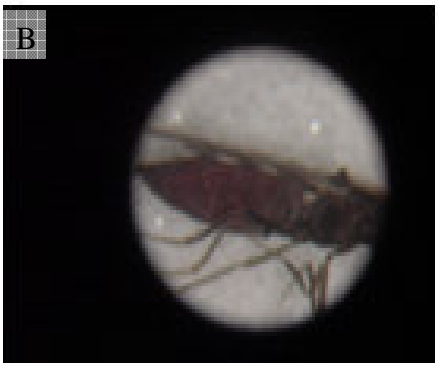
C – Feeding container on top of the blood

D – Feeding container in water bath

Fig. 7 A-D Blood-feeding of field-collected midges



A - Midges on a refrigerated chill table



B – Blood engorged female

C – Sorting of blood engorged females

D – Unwaxed paper cup for the incubation of engorged midges, 10 days at 23.5 °C

Fig. 8 A-D Blood-feeding of field-collected midges

4.2.4 Virus isolation from individual midges

Midges were assayed for virus immediately after feeding on blood-virus mixtures or after 10 days extrinsic incubation at 23.5 ºC. Individual females were homogenized in chilled 90 µl Eagles minimum essential medium (EMEM) using a battery-operated microtissue grinder (Kontes, Vinelan, NY). The final concentration of antibiotics was 500 IU penicillin, 500 µg streptomycin and 1.25 µg fungizone per mL of EMEM. Homogenised samples were centrifuged for 5 minutes at 4 °C, and the supernatants transfered into 0.5 mL microfuge tubes. Ten micro litres of foetal-calf serum (Delta Bioproducts, South Africa) was added, and each sample was then incubated overnight at 4 °C. The following day 20 µl of each insect homogenate was inoculated into each of three microtitre plate wells, and 50 µl of BHK-21 cell suspension in EMEM containing approximately 2 x 10^5 cells/mL, was added. The final concentration of foetal-calf serum in the total volume (75 µl) of the mixture was 5%. The inoculated microplates were incubated at 37 °C in an incubator containing 5% CO_2 and observed microscopically for cytopathic effects (CPE) for four days post inoculation.

All positive sample were titrated using a microtitration assays in BHK-21 cells. Twenty microlitres of the specific midge homogenate was first diluted 1:5 followed by three additional ten-fold dilutions. Of each dilution, 25 µl was transfered into each of three microtitre plates and treated as described above for individual midges.

4.2.5 Virus identification and statistical analysis

The identity of orbivirus isolates was determined by a micro-titre virus-neutralisation procedure (House *et al.* 1990), using type-specific antisera produced in guinea pigs (Katz *et al.* 1993). Virus concentrations calculated by the method of Kärber (1931), were expressed as $\log_{10}TCID_{50}$/midge.

Statistical differences between experimental groups were analyzed using Fisher's exact test and/or Chi-squared analysis. *P*-values of <0.05 were used as the cut-off for statistical significance.

4.3 Results and discussion

The experimental procedure for and average success rate in determining oral susceptibility in field-collected midges are summarized in Fig. 9. The overall success rate (10%) as depicted in Fig. 9 highlights the necessity of using relative large collection of field populations for oral susceptibility studies.

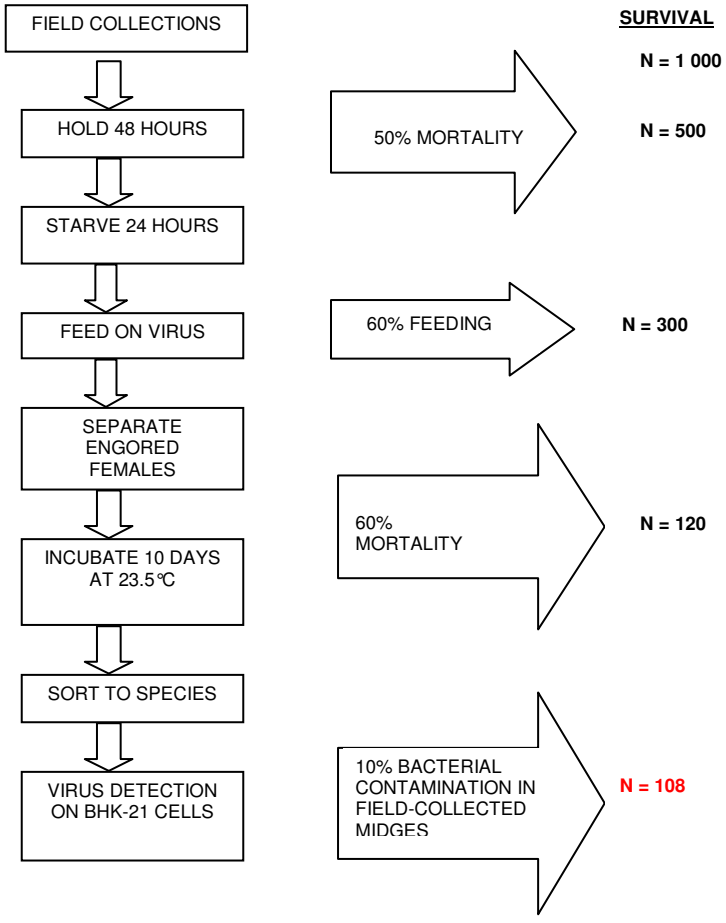


Fig. 9 Experimental procedure for and average survival rate in determining oral susceptibility in field-collected midges. (Adapted from Standfast *et al.* 1992)

4.3.1 Bluetongue virus reference strains

The feeding rates for field-collected *Culicoides* females varied from 10 to 70%. A total of 9 410 midges were fed in 28 attempts on 13 different serotypes of BTV of which only 4 535 (48.2%), representing 19 *Culicoides* species, survived the 10 day incubation period at 23.5 °C. Of the 13 BTV serotypes used, seven (BTV-1, -10, -11, -12, -13, -16 and -19) were recovered from *C. imicola* and 11 (BTV-1, -2, -4, -7, -8, -9, -10, -11, -12, -13, -16 and -19) from *C. bolitinos* after 10-day incubation, although the numbers of the latter available for testing, were lower compared to *C. imicola* (Table 4.4). For the same BTV serotypes the virus recovery rates were higher in *C. bolitinos* than in *C. imicola* and for eight serotypes (BTV-1, -7, -9, -10, -11, -12, -16 and -19) these differences were statistically significant (P=<0.05). Despite a relatively high virus titre of BTV-6 in the blood meal, this serotype could not be recovered from either *C. imicola* or *C. bolitinos* after extrinsic incubation. The mean virus titres in the infected *C. imicola* females after 10 days incubation ranged from 1.4 to 2.4, and *C. bolitinos* females from 1.4 to 3.3 $\log_{10}TCID_{50}$/midge (Table 4.4).

The virus titres in the blood meal of the serotypes used ranged from 4.8 (BTV-16) to 7.1 $\log_{10}TCID_{50}$/mL of blood (BTV-19) (Table 4.4). Therefore, it was not possible to statistically compare virus recovery rates from the same *Culicoides* species for all BTV serotypes used. A comparison of virus recovery rates of BTV-1, -8 and -11 (virus titres 6.8 to 6.9 $\log_{10}TCID_{50}$/mL of blood) in *C. imicola* shows that the recovery rates of BTV-1 and -11 were significantly higher than that of BTV-8 (P<0.001) but there was no significant difference (P=0.243) in the virus recovery rate of BTV-1 or -11. Similarly, in *C. bolitinos* the virus recovery rate of BTV-1 (P=0.001) and BTV-11 (P=0.020) were significantly higher than of BTV-8 (Table 4.4). For BTV-2, -7 and -12 (virus titre of the blood meal = 6.1 $\log_{10}TCID_{50}$/mL of blood) no significant differences in the virus recovery rate (X^2=3.074, d.f.=2,

P=0.215) were found in *C. imicola*. In *C. bolitinos*, however, the virus recovery rate of BTV-7 was significantly higher (*P*<0.001) than that of BTV-2 (Table 4.4). Although the virus titre of BTV-16 in the blood meal was relatively low (4.8 $\log_{10}TCID_{50}$/mL of blood), the virus was recovered from seven of 26 specimens of *C. bolitinos* after extrinsic incubation (Table 4.4) and the virus recovery rate was significantly higher than that for viruses fed at higher titres; BTV-2 (*P*=0.001), BTV-4 (*P*=0.020), BTV-9 (*P*=0.037) and BTV-8 (*P*=0.006).

Because of differences in the virus titres of BTV serotypes used to spike blood meals in this study, it was not possible to compare the virus recovery rates for the same *Culicoides* species for all the different viruses. However, where infectivity of BTV serotypes was the same or comparable in blood meals, significant differences were found in the virus recovery rates (Table 4.4). Field populations of *Culicoides* species may vary greatly in respect to susceptibility to BTV infection and it was demonstrated that some field populations of *Culicoides* species might be completely refractory to infection by some serotypes (Ward 1994; Mellor 2004). The relatively low virus recovery rates found in this study in *C. imicola* for most of the BTV serotypes do not necessarily reflect its low vectorial capacity. For example, *C. brevitarsis* is considered the most competent vector of BTV in Australia even though the highest prevalence of infection determined in this species by feeding on viraemic sheep was only 1.5% (Bellis *et al.* 1994). In the field, the low infection rate with BTV in *C. brevitarsis* is likely to be compensated by very high biting rates leading to successful transmission of the virus (Muller 1985; Mullens *et al.* 2004). Similar to the situation in Australia, the high abundance of *C. imicola* in South Africa (Venter *et al.* 1996b; Meiswinkel *et al.* 2004c) at livestock can compensate for its low vector competence demonstrated in this study.

Table 4.4 Virus recovery rates and virus titres of field-collected *Culicoides* midges maintained for 10 days at 23.5°C after feeding on blood containing bluetongue virus

BTV serotype	19	1	8	11	6	9	4	2	7	12	10	13	16
Virus titre in blood meal[1]	7.1	6.9	6.8	6.8	6.6	6.6	6.4	6.1	6.1	6.1	5.8	5.6	4.8
C. imicola													
No. positive/ no. tested (%)	1/130 (0.8)	19/340 (5.6)	0/234	13/155 (8.4)	0/231	0/197	0/203	0/198	0/167	1/119 (0.8)	1/193 (0.5)	1/198 (0.5)	2/232 (0.9)
Mean virus titre/midge[2](STD[3])	1.4	2.4 (0.85)		2.1 (1.55)						2.3	1.9	1.7	2.4 (0)
C. bolitinos													
No. positive/ no. tested (%)	2/12 (16.7)	26/98 (26.5)	1/38 (2.6)	2/4 (50.0)	0/13	5/61 (8.2)	1/49 (2.0)	1/63 (1.6)	10/24 (41.7)	2/4 (50.0)	4/11 (36.4)	0/10	7/26 (26.9)
Mean virus titre/midge (STD)	3.3 (1.24)	2.8 (1.04)	1.4	3.1 (0.06)		2.8 (1.26)	1.7	2.7	2.6 (1.44)	2.3 (0.88)	2.1 (1.54)		3.3 (0.71)
C. enderleini													
No. positive/ no. tested (%)		0/4	0/1		0/2	1/2 (50.0)	0/6	0/10		0/2		0/1	0/2
Mean virus titre/midge (STD)						2.4							
C. zuluensis	0/1	0/10			0/1	0/1	0/4						
No. positive/ no tested (%)													1/2 (50.0)
Mean virus titre/midge (STD)													2.7
C. milnei													
No. positive/ no tested (%)							1/1 (100)						
Mean virus titre/midge (STD)							2.4						

[1]$Log_{10}TCID_{50}$/mL of blood, [2]$Log_{10}TCID_{50}$/midge, [3]Standard deviation

A potential problem with using field-collected *Culicoides* in assessing susceptibility for a virus under laboratory conditions is that some specimens may have been infected with unknown field viruses. However, virus recovery from field-collected insect vector populations is generally very low (Walter *et al.* 1980; Gerry *et al.* 2001). Therefore, the virus recovery rate recorded for *Culicoides* in the present study were most likely the result of laboratory infection. This is supported by the fact that the identities of all BTV serotypes recovered from midges were the same as those used in the experimental infections.

Compared to the Onderstepoort population of *C. imicola,* the Clarens population of *C. bolitinos* was significantly more susceptible to infection with different BTV serotypes and supported their replication to higher titres. *Culicoides imicola* and *C. bolitinos* ingest similarly sized blood meals, and it has been demonstrated that they take up similar amounts of an orbivirus during an artificial feeding (Paweska *et al.* 2002; Venter *et al.* 2005). Results of previous vector competence studies in South Africa have shown that, compared to *C. imicola,* the Clarens population of *C. bolitinos* not only supported replication of BTV-1, -3 and -4 to higher titres (Venter *et al.* 1998), but also had a significantly higher transmission potential for BTV-1 over a range of different incubation periods and temperatures (Paweska *et al.* 2002).

In addition, BTV was recovered from three other *Culicoides* species, namely *Culicoides enderleini* Cornet & Brunhes (BTV-9), *C. milnei* (BTV-4) and *C. zuluensis* (BTV-16); BTV virus titres ranged in the infected midges from 2.4 to 2.7 $\log_{10}TCID_{50}$/midge (Table 4.4). These three *Culicoides* species are relatively abundant in South Africa and are known to attack livestock (Nevill *et al.* 1992b; Venter *et al.* 1996b; Meiswinkel *et al.* 2004c). While the latter two species are widespread in South Africa, *C. milnei* is considered to be localised in its distribution (Nevill *et al.* 1992b). Bluetongue virus has been isolated from field-collected specimens of *C. milnei* in Kenya (Walker & Davies 1971). Based on overall abundance in light traps,

geographical distribution, virus isolation from field-collected *Culicoides*, host preference and larval habitats, Nevill *et al.* 1992b rate both *C. zuluensis* and *C. enderleini* as having a high vector potential; whereas *C. milnei* was considered to have a low vector potential. The possible involvement of non-*Avaritia* species in the transmission of BTV in South Africa was reported by Paweska *et al.* (2002) and reflects findings made elsewhere in the world (Meiswinkel *et al.* 2004a). No virus could also be recovered from 316 specimens, representing a further 14 *Culicoides* species, after incubation (Table 4.5).

Table 4.5 *Culicoides* species from which no virus could be isolated after feeding on blood containing bluetongue virus (BTV) and 10 days extrinsic incubation at 23.5 ^{o}C

BTV serotype	19	1	11	6	9	4	2	12	13	16
Virus titre in blood meal ($log_{10}TCID_{50}$/mL of blood)	7.1	6.9	6.8	6.6	6.6	6.4	6.1	6.1	5.6	4.8
Culicoides species										
C. gulbenkiani		43			26	13	57			
C. engubandei		25			10	14	22			
C. magnus		5			3	21	3			
C. leucostictus	1	4			1	13	6			
C. nivosus		1			2	3	4			6
C. subschultzei		2		2	4	3			1	
C. schultzei		2	1					1		
C. pycnostictus		1			3					
C. bedfordi		1			2				1	
C. coarctatus					2	1				
C. nevilli					2	1				
C. neavei							1			
C. huambensis						1				
C. dutoiti					1					

The virus titres in some individual midges determined in this study after extrinsic incubation were equal to or above 2.5 $log_{10}TCID_{50}$/midge (Table 4.4). Titres of this magnitude of an orbivirus have been postulated as an indicator of a fully

disseminated “transmissible” infection (Jennings & Mellor 1987). Since this infectious threshold helps to distinguish between transmissive and non-transmissive individuals, results of laboratory infection studies can provide data of epidemiological importance. However, this threshold was derived from the *C. sonorensis*/BTV laboratory model and it is unlikely that it can be directly applied to other *Culicoides* species, especially those of smaller size than *C. sonorensis.* The higher mean virus titres found in *C. bolitinos* after incubation can, however, be indicative of a higher transmission potential in this species.

The results of this study confirm that the Clarens population of *C. bolitinos* is significantly more susceptible to oral infection with different serotypes of BTV than the Onderstepoort population of *C. imicola*. Should these results reflect the same level of genetic susceptibility to infection with this virus in other populations of *C. imicola* and *C. bolitinos*, the role of the latter in BTV transmission in South Africa might be considerable, despite its general lower abundance compared to *C. imicola.* Nevertheless, present results contribute to a better understanding of BT epidemiology in cooler areas of South Africa where the known vector of BTV, *C. imicola,* is virtually absent.

4.3.2 Bluetongue live-attenuated vaccine strains

During the summer of 2002/3 the live-attenuated vaccine strains of BTV-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -16 and -19 were used in oral susceptibility studies. A total of 5 359 (54.5%) *Culicoides* females survived the 10 day incubation period at 23.5°C. The number of midges assayed successfully after incubation are shown in Table 4.6. Virus recovery results in midges, which have survived the 10 day incubation, indicated that at least 11 (BTV-1, -3, -4, -5, -6, -7, -8, -10, -12, -13 and -19) of the 16 live-attenuated BTV strains used had replicated in *C. imicola*. Lower numbers of *C. bolitinos* were tested and virus recovery results from incubated midges

indicated that seven (BTV-1, -3, -4, -5, -6, -10 and -12) of the 16 live-attenuated BTV vaccine strains used, replicated in *C. bolitinos* (Table 4.6). While BTV-2, -9, -11, -14 and -16 were not recovered from either *C. imicola* or *C. bolitinos,* BTV-7, -8, -13 and -19 were only recovered from *C. imicola* in (Table 4.6). Virus recovery rates, ranging from 62.5% (BTV-5) to 3.0% (BTV-4) in *C. bolitinos,* were higher than those in *C. imicola* were. For BTV-1 (P=0.003), BTV-3 (P=0.036), BTV-5 (P<0.001) and BTV-12 (P=0.006) virus recovery rates from *C. bolitinos*, compared to *C. imicola*, were statistically significantly higher.

The virus titres in the blood meal of the serotypes used in this study ranged from 4.6 (BTV-8) to 6.6 $\log_{10}TCID_{50}$/mL of blood (BTV-5) (Table 4.6) and it is therefore not possible to compare virus recovery rates from the same *Culicoides* species for all the BTV serotypes. A comparison of virus recovery rates of BTV-1, -3, -6 and -16 (virus titre 6.1 $\log_{10}TCID_{50}$/mL of blood) showed that the recovery rates of these serotypes were not significantly different in *C. imicola* (Table 4.6). In *C. bolitinos* the virus recovery rate of BTV-1 was significantly higher than that of BTV-16, (P=0.018) (Table 4.6). A comparison of virus recovery rates of BTV-2, -10, -13 and -19 (virus titre of the blood meal = 5.8 $\log_{10}TCID_{50}$/mL of blood) in *C. imicola* showed that the recovery rate of BTV-10 was significantly higher than that of BTV-2 (P=0.004). Similar comparisons of the recovery rates of BTV-9 and -12 (virus titre of the blood meal = 5.3 $\log_{10}TCID_{50}$/mL of blood) showed that the recovery rate of BTV-12 was significantly higher in both *C. imicola* (P=0.027) and *C. bolitinos* (P=0.014). Although the virus titre of BTV-8 in the blood meal was relatively low (4.6 $\log_{10}TCID_{50}$/mL of blood), the virus was recovered from nine of 142 specimens of *C. imicola* after extrinsic incubation (Table 4.6) and the virus recovery rate was significantly higher than for those viruses fed at higher titres; BTV-1 (P=0.009), BTV-2 (P<0.001), BTV-3 (P=0.007), BTV-4 (P=0.001), BTV-6 (P=0.001), BTV-9

(P<0.001), BTV-11 (P=0.002), BTV-12 (P=0.037), BTV-13 (P=0.022), BTV-14 (P<0.001), BTV-19 (P=0.023) and BTV-16 (P<0.001).

Table 4.6 Virus recovery rates and titres of field-collected *Culicoides* midges maintained for 10 days at 23.5 ^{o}C after feeding on blood containing vaccine strains of bluetongue virus (BTV)

BTV serotype	Virus titre in bloodmeal[1]	*C. imicola* %	Mean virus titre/midge[3] (STD) Range	*C. bolitinos* %	Mean virus titre/midge (STD) Range	*C. enderleini* %	Virus titre/midge
5	6.6	50/473[2] 10.6	3.9 (1.204) 1.1-6.2	10/16 62.5	4.4 (1.261) 2.4-6.2	1	
4	6.3	3/342 0.9	2.0 (0.774) 1.1-2.4	1/33 3.0	3.4		
1	6.1	7/402 1.7	1.3 (0.733) 0.7-2.4	3/13 23.1	1.6 (0.698) 1.1-2.4	1	
3	6.1	1/165 0.6	4.2	1/3 33.3	1.7	1/5 20	4.7
6	6.1	1/226 0.4	2.4	1/11 9.1	2.4		
16	6.1	0/218		0/34			
2	5.8	0/291		0/7			
10	5.8	6/200 3.0	1.3 (0.602) 0.7-1.9	1/22 4.5	2.2	3	
13	5.8	1/125 0.8	1.9	0/12			
19	5.8	1/122 0.8	1.9	0/13			
7	5.6	4/164 2.4	3.0 (0.595) 2.4-3.7	0/18			
9	5.3	0/343		0/55			
12	5.3	4/234 1.7	2.2 (1.816) 0.7-4.4	5/43 11.6	3.7 (1.022) 2.4-4.7		
14	5.1	0/190		0/6			
11	4.8	0/146		0/29		1	
8	4.6	9/142 6.3	3.2 (1.710) 0.7-4.9	0/18		1	

[1]$Log_{10}TCID_{50}$/mL of blood, [2]Number positive/number tested, [3]$Log_{10}TCID_{50}$/midge

One of five specimens of a non-*Avaritia* species, namely *C. enderleini* which had fed on BTV-3 assayed positive for this serotype after incubation. The virus titre in this midge was 4.7 $log_{10}TCID_{50}$/midge (Table 4.6). Based on abundance in light traps, geographical distribution, host preference and larval habitat, Nevill *et al.* (1992b) rated *C. enderleini* as having a high potential vector capacity and this work tends to support

that statement. The possible involvement of non-*Avaritia Culicoides* species in the transmission of BTV in South Africa has been reported on previously (Paweska *et al.* 2002) and reflects similar findings made elsewhere in the world where such species may be of major importance in the transmission of BTV (Meiswinkel *et al.* 2004a). No virus could be recovered from an additional 14 *Culicoides* species assayed (Table 4.7).

Table 4.7 *Culicoides* species from which no virus could be isolated after feeding on blood containing vaccine strains of bluetongue virus (BTV) after 10 days extrinsic incubation at 23.5 ^{o}C

BTV serotype	5	4	1	3	6	16	2	10	13	19	7	9	12	14	11	8
Virus titre of bloodmeal ($log_{10}TCID_{50}$/mL of blood)	6.6	6.3	6.1	6.1	6.1	6.1	5.8	5.8	5.8	5.8	5.6	5.3	5.3	5.1	4.8	4.6
Culicoides species			10	2	1	5	4	2	2		2			1		1
C. nivosus																
C. pycnostictus		7	2	3		6	2	1		1		3				2
C. leucostictus		7	3		1	2	3	1				5	1	1		1
C. zuluensis		2	5	7	1	2		2		1	1	3				
C. subschultzei		2	1				2						2			
C. neavei		1	1				1			1						
C. coarctatus			2			1										
C. gulbenkiani			1			1										1
C. engubandei			1											1		
C. bedfordi		1										1				
C. schultzei			1													
C. nigripennis															1	
C. magnus						1										
C. nevilli	1															

The present study showed that *C. imicola* and *C. bolitinos* can become infected and replicate at least 12 of the 16 attenuated BTV strains used in current production of commercial polyvalent vaccines in South Africa. The relatively low virus recovery rates found in *C. imicola* for most of the highly attenuated serotypes used is comparable to that showed for various other-low passaged BTV serotypes (Venter *et al.* 1998; Paweska *et al.* 2002, Table 4.4). As discussed for the BTV

reference strains, low virus recovery rates of BTV from *C. imicola* do not necessarily reflect its low vectorial capacity.

Because of differences in the virus titres of the BTV serotypes used to spike blood meals in this study, it was not possible to compare the virus recovery rates for the same *Culicoides* species for all the different viruses. However, where infectivity of BTV serotypes was the same or similar, such comparisons could be made and significant differences were sometimes found in the virus recovery rates (Table 4.6). The significance of this is not clear but field populations of *Culicoides* species may vary greatly in genetic susceptibility to BTV infection. Indeed it has already been demonstrated that some populations of a species might be completely refractory to infection with some serotypes of the virus (Ward 1994; Mellor 2004; Carpenter *et al.* 2006). In the present study, using populations of *C. imicola* that have shown to express low levels of competence for BTV, it may be that the differences recorded relate only to the probability of a competent individual being included in the relatively small sample sizes and surviving the incubation period.

Only attenuated viruses which yielded a viraemia of not more than 3 $\log_{10}$PFU[7]/mL of blood in sheep are used as vaccine strains for routine immunization (Dungu *et al.* 2004a,b). The minimum level of viraemia that is necessary to infect South African *Culicoides* vectors with BTV is unknown. Based on the blood meal size of *C. imicola* and *C. bolitinos,* which ranges from 0.01 to 0.06 µl, it can be calculated that a viraemia of 5 $\log_{10}$ID[8]/mL of blood would be required to expose each vector to approximately one $TCID_{50}$ of virus (Venter *et al.* 2005). Muller *et al.* (1982) reported that the volume of the blood meal taken by a single *C. brevitarsis* as about 0.03µl. These authors were able to demonstrate an infection rate of 0.2% in midges fed on cattle with a BTV-20 viraemia of 2 $\log_{10}TCID_{50}$/mL of blood. This approximates to infection with one $TCID_{50}$ of virus per midge. Bonneau *et al.* (2002) succeeded in

[7] Plaque forming units
[8] Infective dose

infecting *C. sonorensis* when feeding this species on a BTV infected sheep at a time when viraemia was not detectable at all. This indicates that any titre of viraemia may be sufficient to establish an infection in a proportion of biting vectors. The number of infected individuals will depend upon the biting rate and the competence rate of the species involved. Vaccine strains used in this study have recently been reported to cause a viraemia in Dorset Poll sheep that persisted for up to 17 days, and ranged from 2.5 to 6.3 $\log_{10}TCID_{50}$/mL of blood. Such levels of viraemias are sufficient to infect *Culicoides* vectors (Veronesi *et al.* 2005).

In this study the virus titres recorded indicated that virus had replicated to high levels in at least some of the infected midges (Table 4.6). As mentioned earlier concentration of live virus in the head or body of a midge higher than 2.5 $\log_{10}TCID_{50}$/midge has been postulated as an indicator of a fully disseminated infection (Jennings & Mellor 1987). This threshold value was derived from *C. sonorensis*-BTV laboratory-based model and it is uncertain if it can be used for interpretation of vector competence results in other, smaller, *Culicoides* species. The genus *Culicoides* is biologically highly diverse and thus extrapolation of vector competence data from one species to others is not recommended (Tabachnick 1992). Nevertheless, the results of the present study indicate that transmission of the attenuated vaccine strains of BTV by at least a proportion of infected *C. imicola*, might be possible. The higher mean virus titres found in *C. bolitinos* after incubation was indicative of a higher transmission potential in this species.

In South Africa the prevalence of infections in field-collected vector populations, even in outbreak situations, is generally very low, <0.06% (Nevill *et al.* 1992b; Wieser-Schimpf *et al.* 1993; Gerry *et al.* 2001; Meiswinkel & Paweska 2003; Venter *et al.* 2006). Therefore, the virus recovery rates in this study, ranging between 0.6% and 62.5%, for *C. imicola* and *C. bolitinos,* were most likely the result of laboratory infection. This is supported by the fact that the identity of all the virus

isolates recovered from the experimental midges was confirmed as being the serotype of BTV used for infection.

Although vector competency tests provide important information about a specific vector population, they provide little predictability about the behaviour of other populations with the same or different strains of virus (Tabachnick 1992). It should be recognized, therefore, that vector species and different populations within a species vary greatly in respect to susceptibility to orbivirus infection (Ward 1994; Carpenter *et al.* 2006). Some field populations of a vector species may be completely resistant to infection for certain serotypes (Ward 1994; Carpenter *et al.* 2006). Several factors, including population size, infection rate, feeding rate, host preference, survival rates and age of the midges determine how efficiently a *Culicoides* vector population will transmit a virus. Usually, large numbers of vectors are required before virus transmission becomes sustainable because infection rates are very low (Muller *et al.* 1982; Mullens *et al.* 2004).

Considering the very high abundance of the two competent orbivirus vectors in South Africa, *C. imicola* and *C. bolitinos* (Venter *et al.* 1996b; Meiswinkel 1997; Meiswinkel *et al.* 2004c*)*, the reported levels of viraemia in vaccinated animals (Dungu *et al.* 2004a, Veronesi *et al.* 2005) and present results, it seems likely that transmission of BTV vaccine strains by *Culicoides* from vaccinated to unvaccinated animals may occur under field situations.

4.3.2.1 Vector passaged vaccine strains of bluetongue virus

During the summer of 2003/4 field-collected *C. imicola* were fed on either the vaccine strains of BTV-1, -2, -4, -9 and -16 or on vector passaged BTV isolates of the same strains. A total of 3 162 individuals (45.2%) survived the 10 day incubation period at 23.5°C. The number of midges assayed after incubation, the number of passages on

Ae. albopictus C6/36 cell cultures, the titre of the blood meals and the virus recovery rate from midges fed with the various virus isolates are shown in Table 4.8.

Table 4.8 Oral susceptibility of field-collected *C. imicola* to vaccine strains and vector-passaged vaccine strains of bluetongue virus (BTV). Midges tested after 10 days incubation at 23.5 °C

BTV serotype	1		2		4		9		16	
Isolate	A[1]	MI(2)[2]	A	MI(4)	A	MI(4)	A	MI(3)	A	MI(4)
Virus titre of blood meal[3]	6.1	6.3	5.8	6.1	6.3	6.6	5.3	6.8	6.1	5.8
Number positive/ number tested	7/244	0/268	1/168	1/187	0/304	1/265	2/170	2/455	1/374	1/157
% testing positive	2.9		0.6	0.5		0.4	1.2	0.4	0.3	0.6
Mean virus titre/ midge[4]	1.2		0.7	1.4		1.4	1.2	1.1	0.7	1.4
Range	0.7-1.7						0.7-1.7	0.7-1.4	0.7	

[1]Attenuated virus strain, [2]Midge isolate and number of passages on *Ae. albopictus* C6/36 cells, [3]$Log_{10}TCID_{50}$/mL of blood, [4]$Log_{10}TCID_{50}$/midge

The virus recovery rate was significantly higher in *C. imicola* fed on the vaccine strain of BTV-1 (P=0.005) than on an isolate of the same virus passaged twice through *C. imicola* and twice through *Ae. albopictus* C6/36 cell cultures (Table 4.8). There was no significant difference in the virus recovery rates from *C. imicola* fed on the vaccine strains of BTV-2 (P=1.000), BTV-4 (P=0.466), BTV-9 (P=0.299) and BTV-16 (P=0.504) compared to those from *C. imicola* fed with the same viruses that had been subjected to vector/insect cell passages prior to feeding. This suggests that passaging in a vector type of cells did not result in the selection of progeny virus populations with higher infectivity for vectors.

4.3.2.2 Inter-seasonal stability in oral susceptibility in *C. imicola* to bluetongue virus

Vaccine strains of BTV-1, -4, -9 and -16 were used for the oral infection of field-collected *C. imicola* for three consecutive summers. Virus recovery results from midges fed in the 2002/3 and the 2003/4 seasons were compared with each other and with the published results from the 2001/2 season (Venter *et al.* 2004). There were no significant differences in the virus recovery rates between the three seasons (P>0.07 in all cases) analysed (Table 4.9).

Table 4.9 Comparison of virus recovery rates, after 10 days incubation at 23.5 °C, in *C. imicola* fed on the live-attenuated vaccine strains of bluetongue virus (BTV) over three consecutive summer seasons

Season	2002 (Venter *et al.* 2004)	2003	2004
BTV-1 (6.1 $\log_{10}$TCID$_{50}$/mL of blood) Number positive/number tested (%)	22/712 (3.1)	7/402 (1.7)	7/244 (2.9)
BTV-4 (6.3 $\log_{10}$TCID$_{50}$/mL of blood) Number positive/number tested (%)	10/886 (1.1)	3/342 (0.9)	0/304
BTV-9 (5.3 $\log_{10}$TCID$_{50}$/mL of blood) Number positive/number tested (%)	0/349	0/343	2/170 (1.2)
BTV-16 (6.1 $\log_{10}$TCID$_{50}$/mL of blood) Number positive/number tested (%)	3/728 (0.4)	0/218	1/374 (0.3)

Inter-seasonal stability in vector competence has been noted for field-collected *C. sonorensis* infected with BTV-10 (Gerry *et al.* 2001). The ability of *C. sonorensis* to support viral replication has shown to be at least partially under genetic control (Tabachnick 1991), which may make this trait relatively stable within particular vector populations over time. Virus recovery from *C. imicola* fed on the vaccine strain of BTV-1 was significantly higher than from midges fed on the vaccine strains of BTV-4 (X^2=12.970, d.f.=1, P<0.001), BTV-9 (X^2=16.929, d.f.=1, P<0.001)

and BTV-16 (X^2=23.509, d.f.=1, P<0.001). Although the virus titre in the blood meal for the vaccine strain of BTV-9 was one log lower than for the other strains there was no significant differences in the virus recovery rates from *C. imicola* fed on BTV-4, -9 and -16 (X^2= 5.870, d.f.=2, P= 0.053) (Table 4.9).

4.3.3 Wild-type and live-attenuated vaccine strains of African horse sickness virus serotype 7

Of 4 131 *Culicoides* fed, a total of 2 407 (58.3%) survived the 10 day incubation period at 23.5°C. The number of midges assayed immediately after feeding and after incubation as shown in Table 4.10 was dependent on the number and species present in the field and on the survival of these species under laboratory conditions. African horse sickness virus serotype 7 was isolated from 31 of 61 (50.8%) *C. imicola* and from 15 of 27 (55.6%) *C. bolitinos* assayed immediately after feeding.

Table 4.10 Recovery of African horse sickness virus serotype 7 (AHSV-7) field isolates and AHSV-7 attenuated vaccine strain (AHS 31/62) from *Culicoides* species after 10 days extrinsic incubation at 23.5 ^{o}C

AHSV-7 isolate	31/62[2]	23/98	1/99	3/99	7/99	29/99
Virus titre in blood meal ($log_{10}TCID_{50}$/mL of blood)	7.6	7.6	8.2	8.5	7.1	7.1
Culicoides species						
C. imicola	18/135[1]	0/239	1/159	1/222	34/358	0/130
(%)	13.3	0.0	0.6	0.5	9.5	0.0
C. bolitinos	8/192	0/2	1/66	1/110	0/6	0/83
(%)	4.2	0.0	1.5	0.9	0.0	0.0
C. bedfordi	1/6					
(%)	16.7					
C. dutoiti	½			0/3		
(%)	50.0			0.0		
C. engubandei	0/20		1/1	0/1		
(%)	0.0		100	0.0		
C. gulbenkiani	0/4		0/4	1/6		
(%)	0.0		0.0	16.7		

[1]Number positive/number tested, [2]Paweska *et al*. (2003)

In *C. imicola* assayed after 10 days extrinsic incubation the highest virus recovery rate was for the attenuated vaccine isolate 31/62 of AHSV-7 (Table 4.10). The virus recovery rate of the AHSV-7 attenuated strain was significantly higher than that of the AHSV-7 23/98 (Pietermaritzburg), AHSV-7 1/99 (Krugersdorp), AHSV-7 3/99 (Derdepoort) and AHSV-7 29/99 (Rustenburg) isolates ($P<0.001$ in all cases). There was no significant difference in virus recovery rate for AHSV-7 vaccine strain (13.3%) and AHSV-7 7/99 (Onderstepoort) (9.5%) ($P=0.249$). Two isolates, AHSV-7 23/98 (Krugersdorp) and AHSV-7 29/99 (Rustenburg), could not be recovered from 239 and 130 *C. imicola* individuals respectively assayed after incubation (Table 4.10).

The numbers of *C. bolitinos* assayed after incubation were lower than that of *C. imicola* and three of the field isolates used could not be recovered from this species (Table 4.10). Similar to *C. imicola* the highest virus recovery rate in *C. bolitinos* was for the attenuated vaccine strain of AHSV-7 (AHSV-7 31/62) but it was not significantly different from AHSV-7 23/98 (Pietermaritzburg) ($P=1.000$), AHSV-7 1/99 (Krugersdorp) ($P=0.455$), AHSV-7 3/99 (Derdepoort) ($P=0.163$), AHSV-7 7/99 (Onderstepoort) ($P=1.000$).or AHSV-7 29/99 (Rustenburg) ($P=0.111$).

The virus recovery rate of the AHSV-7 attenuated vaccine strain (AHSV-7 31/62) was significantly higher in *C. imicola* than in *C. bolitinos* ($P=0.003$). No significant difference in the virus recovery rates from *C. imicola* and *C. bolitinos* were found for AHSV-7 1/99 (Krugersdorp) ($P=0.506$), AHSV-7 3/99 (Derdepoort) ($P=0.554$) and AHSV-7 7/99 (Onderstepoort) ($P=1.000$). Vaccine strain was also recovered from one of six specimens of *Culicoides* (Unplaced) *bedfordi* Ingram & Macfie and from one of two *Culicoides* (*Synhelea*) *dutoiti* de Meillon (Table 4.10). In addition, AHSV-7 1/99 (Krugersdorp) was recovered from a single specimen of *Culicoides* (*Pontoculicoides*) *engubandei* de Meillon and AHSV-7 3/99 (Derdepoort) from one of six *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro (Table 4.10). Both *C. gulbenkiani* and *C. engubandei* have been shown to feed on horses (Meiswinkel *et*

al. 2004c). No virus could be recovered from 12 other *Culicoides* species assayed (Table 4.11).

Table 4.11 *Culicoides* species from which neither African horse sickness virus serotype 7 (AHSV-7) field isolates nor the AHSV-7 attenuated vaccine strain (AHS 31/62) could be recovered after 10 days extrinsic incubation at 23.5 °C

AHSV isolate	31/62[1]	23/98	1/99	3/99	7/99	29/99
Virus titre in blood meal ($log_{10}TCID_{50}$/mL of blood)	7.6	7.6	8.2	8.5	7.1	7.1
Culicoides species						
C. coarctatus	4		2	2		
C. enderleini	2					
C. huambensis				2		
C. leucostictus	3		1	3	1	1
C. magnus	21		8	14		1
C. neavei					1	
C. nevilli	1					
C. nivosus			1		5	
C. onderstepoortensis	2			1		
C. pycnostictus	3			6	2	
C. subscultzei			1			
C. zuluensis	4	1	1	15	7	7

[1]Paweska *et al*. (2003)

This study demonstrated generally higher recovery rates for AHSV-7 vaccine strain than for field isolates of the same serotype, and the fact that vaccine virus was recovered from *Culicoides* belonging to different subgenera, increases safety risks associated with the use of live-attenuated vaccines. Live-attenuated vaccines should be safe, avirulent and efficacious and should not revert to virulence in vaccinated animals or be transmitted from vaccinated to susceptible hosts by insect vectors. The latter criterion is very important because insect-mediated transmission of an attenuated virus from vaccinated to susceptible animals, with subsequent replication in mammalian host species increases the possibility of reversion to virulence. The potential risk also

includes vaccine virus reassortment with wild-type virus, which may generate virus strains with new virulence characteristics (Riegler *et al.* 2000; Riegler 2002).

Paweska *et al*. (2003) showed that South African *Culicoides* species are susceptible to oral infection with live-attenuated AHSV strains currently used in the production of the Onderstepoort polyvalent vaccine. These authors also demonstrated that the AHSV-7 vaccine strain in *C. imicola*, *C. bolitinos*, *C. bedfordi* and *C. dutoiti*, and the AHSV-4 vaccine strain in *C. bolitinos* replicated to a titre which in previous studies using the closely related BTV and AHSV in *C. sonorensis* (Jennings & Mellor 1987; Riegler 2002), indicated fully disseminated infections and, by analogy, would therefore be sufficient for transmission by these species when feeding on susceptible equids. The results of this study indicate a significant lower oral susceptibility of *C. imicola* to field isolates of AHSV-7 than to the attenuated AHSV-7 vaccine strain (Table 4.10). The attenuated AHSV-7 vaccine strain has been intensively used in the current formulation of the polyvalent vaccine for more than three decades and was shown to replicate to potentially transmissible levels in the most abundant and widespread, livestock-associated *Culicoides* species in South Africa, namely *C. imicola* and *C. bolitinos.* Thus, significantly lower recovery rates for field isolates representing wild-type AHSV-7 than for AHSV-7 vaccine strain might indicate that the latter had successfully co-adapted to *Culicoides* vectors over the long period of its wide and intensive use for immunisation of horses in South Africa. In this respect, it is worth mentioning that sequencing analysis of VP2 genes of AHSV-2 suggest a very close genetic relationship between the AHSV-2 isolates recovered in 2002 from clinical cases in distinct geographical areas in South Africa and the current AHSV-2 vaccine strain (Paweska *et al*. 2003). The AHSV-2 vaccine strain represents a culture-attenuated 82/61 strain of AHS isolated in South Africa in 1961 (Erasmus 1965).

Previous studies have demonstrated that *C. bolitinos* is more susceptible to oral infection with BTV and supported replication of several serotypes to significantly higher

titres than *C. imicola* (Venter *et al.* 1998; Paweska *et al.* 2002). In the present study *C. imicola* was shown to be only more susceptible to infection with the attenuated vaccine strain of AHSV-7 then *C. bolitinos.* Paweska *et al.* (2003) reported recovery of live-attenuated vaccine strains of AHSV from experimentally infected six Old World livestock-associated non-*Avaritia Culicoides* species.

The highest virus recovery rate from the Onderstepoort population of *C. imicola* of an AHSV-7 strain isolated from a naturally infected horse at Onderstepoort (Table 4.10) and the significant differences found in the oral susceptibility of *C. imicola* to AHSV-7 isolates originating from different geographical localities, might indicate a higher fitness between the virus and *Culicoides* vectors from the same geographical locality. The practical implication of the possible co-adaptation of endemic viruses and *Culicoides* species within geographically distinct areas is that a true assessment of vector competence and capacity might be elusive and even more complicated to study if it would require some level of real-time monitoring, e.g. testing local *Culicoides* populations using currently circulating variants of orbiviruses.

Live-attenuated viruses used for vaccination against AHS should not generate viraemia in susceptible horses higher than 3 $\log_{10}$ PFU/mL of blood (Dungu *et al.* 2006). In most vaccinated horses viraemia cannot be detected by traditional isolation techniques but some individuals may be viraemic for 3 to 5 days. In addition, the current infectivity criterion used to evaluate vaccine safety is practically based on results in a very limited number of horses, thus one cannot exclude the possibility that during massive annual vaccination some individual animals may develop higher levels of viremia (Paweska *et al.* 2003). In experimentally infected horses with wild-type AHSV, virus titres may be as high as 7.5 $\log_{10}TCID_{50}$/mL of blood (Scanlen *et al.* 2002) and viraemia may last 2 to 3 weeks (Paweska *et al.* 2003). Although, this clearly demonstrates that the tissue-culture attenuation used for the production of current

AHSV vaccines decreases the level of viraemia in horses, results of this study demonstrate that the attenuation process does not decrease the ability of AHSV to replicate in vector-competent midges.

4.3.4 *Culicoides* population susceptibility to infection with African horse sickness virus

A total of 13 AHSV isolates were used to determine and compare the oral susceptibility of *Culicoides* populations at Clarens and at the ARC-OVI. All nine reference strains of AHSV as well as three of the most recent field isolates were included and the reference strain of AHSV-5 was used at two titres (Table 4.12). Between November 2005 and April 2006 5 438 midges collected at the ARC-OVI were fed in 20 feeding attempts on 13 AHSV isolates. A total of 2 879 (52.9%) midges survived the incubation period. In the same period 15 050 midges collected at Clarens were fed in 37 feeding attempts on the identical isolates and 4 510 (30.0%) midges survived the 10 day incubation period at 23.5 °C. The number of midges tested from each location and the virus recovery rates for the *Culicoides* species assayed are shown in Table 4.12. The virus titres in the infected midges are shown in Table 4.13.

Similar to the results obtained for BTV, significant differences were obtained in the virus recovery rates of the various isolates of AHSV from *C. imicola* collected at Onderstepoort. Three of the AHSV isolates (the reference strain of AHSV-1 and the field isolates of AHSV-2 (4/04 Grahamstown) and AHSV-7 (2/04 Lephale)) could not be isolated from *C. imicola* (Table 4.12). The highest virus recovery rate (20.2%) in *C. imicola* collected at Onderstepoort was that of reference strain of AHSV-7 (Table 4.12). The virus recovery was significantly higher than that of the reference strains of AHSV-2, -3, -4, -5, -6, -9 and the field isolate of AHSV-9 (32/04 Mokopane) ($P<0.001$ in all cases). It was also significantly higher than that of the reference strain of AHSV-8 ($P=0.002$). In evaluating these results it must be taken in consideration

that the virus titre in the infective blood meal for AHSV-7 (6.1 $\log_{10}$TCID$_{50}$/mL of blood) was at least one log lower than that of the reference strains of AHSV-5 and AHSV-9 (Table 4.12).

For *C. imicola* collected at Clarens the highest recovery rate (40.6%) was that of the reference strain of AHSV-6 (Table 4.12). The virus recovery rate was significantly higher than that of the reference strains of AHSV-5, -6, -8, -9 (P<0.001 in all cases), AHSV-3 (P=0.024) and the field isolate of AHSV-9 32/04 (P=0.007). It was, however, not significantly different from that of reference strains of AHSV-2 (P=0.205), AHSV-4 (P=0.650) and AHSV-7 (P=0.278). The virus titre in the infective blood meal for AHSV-6 was at least one log higher than that of most of the other AHSV isolates used (Table 4.12).

More remarkable is that virus recovery rates of identical AHSV isolates from *C. imicola* collected at Clarens were higher than that of *C. imicola* collected at Onderstepoort (Table 4.12). For the reference strains of AHSV-2 (P<0.001), AHSV-5 (P=0.005), AHSV-6 (P<0.001) and AHSV-9 (P=0.027) it was significantly higher. In the United States it was shown that *Culicoides* susceptibility to infection with BTV varies according to geographic area within and between *C. variipennis* (= *C. sonorensis*) populations (Tabachnick 1996). This is now also shown for *C. imicola* in South Africa, with significantly differences being found in the susceptibility of geographically distinct *C. imicola* populations.

The virus concentration in infected *C. imicola* varied from 0.7 to 4.4 $\log_{10}$TCID$_{50}$/midge (Table 4.13). Relative high virus titres (>2.5 $\log_{10}$TCID$_{50}$/midge) in individual midges assayed indicate virus replication and that transmission to susceptible horses may be possible.

Table 4.12 Virus recovery rates in field-collected *Culicoides* midges from either Clarens or Onderstepoort maintained for 10 days at 23.5°C after feeding on blood containing African horse sickness virus

AHSV serotype and isolate (virus titre in blood meal[1])	O[2]	*C. imicola* (%)	*C. bolitinos* (%)	*C. engubandei* (%)	*C. gulbenkiani* (%)	*C. zuluensis* (%)	*C. leucostictus* (%)	*C. magnus* (%)	*C. enderleini* (%)	*C. pycnostictus* (%)	*C. exspectator* (%)	*C. brucei* (%)
1 reference (6.1)	C[3]	4	1/85[5] (1.2)	3	4	1	1/22 (4.5)	5				
	OP[4]	159	1				1					
2 4/04 (5.4)	C	6	168	16	34	6	14	42		10		
	OP	190	1				2	1		1		
2 reference (5.8)	C	4/17 (23.5)	9/75 (12.0)	1/1 (100)	2/6 (33.6)	1	19/50 (38.0)	22		2	1/2 (50.0)	
	OP	2/292 (0.7)	1/2 (50.0)				1		10			
3 reference (6.3)	C	8	3/18 (16.7)	2	2	2	4/29 (13.8)	3/5 (60.0)	1/1 (100)	2		
	OP	2/142 (1.4)					1		1			
4 reference (6.1)	C	1/4 (25.0)	1/24 (4.2)	3	1		15	1		2		
	OP	11/223 (4.9)	1			2						
5 reference (7.8)	C	120	19		2		2					
	OP	1/423 (0.2)										
5 reference (8.8)	C	42/406 (10.3)	5/118 (4.2)	4	9	17	25	6		3		
	OP	6/175 (3.4)										
6 reference 8.1	C	106/261 (40.6)	11/128 (8.6)	3		13	6	1/15 (66.7)				
	OP	12/193 (6.2)				1						
7 2/04 (6.2)	C	2	98	19	10	1	9	11				
	OP	159							1			
7 reference (6.1)	C	24/73 (32.9)	20	1	5		22					
	OP	21/104 (20.2)					2					
8 reference (5.8)	C	32/339 (9.4)	1/54 (1.9)	2	2	8	27	7		1		
	OP	7/113 (6.2)					1/1 (100)					
9 32/04 (6.2)	C	10	1/87 (1.1)	5	12	8	53	22		8		
	OP	4/161 (2.5)				1	1			1		
9 reference (7.8)	C	38/328 (9.8)	4/81 (4.9)	2/6 (33.3)	8	2/10 (20.0)	4/89 (4.5)	3/20 (15.0)		1/7 14.3)		1/1 (100)
	OP	1/54 (1.9)								1		

[1]$Log_{10}TCID_{50}$/mL of blood, [2]Origin of *Culicoides* [3]Clarens, [4]Onderstepoort, [5]Number positive/number tested

Table 4.13 Virus titres in field-collected *Culicoides* midges from either Clarens or Onderstepoort maintained for 10 days at 23.5°C after feeding on blood containing African horse sickness virus

AHSV serotype and isolate (titre in blood meal[1])	O[2]	*C. imicola*	*C. bolitinos*	*C. engubandei*	*C. gulbenkiani*	*C. zuluensis*	*C. leucostictus*	*C. magnus*	*C. enderleini*	*C. pycnostictus*	*C. exspectator*	*C. brucei*
1 reference (6.1)	C[5]		1 (0.7)				1 (2.4)					
2 reference (5.8)	C	4[3] (1.2[4])	9 (1.0)	1 (0.7)	2 (0.7)		19 (1.7)				1 (0.7)	
Range in virus titre			0.7-1.4				0.7-3.7					
	OP[6]	2 (1.4)	1 (2.4)									
3 reference (6.3)	C		3 (1.3)				4 (1.4)	3 (2.1)	1 (1.4)			
Range in virus titre			0.7-2.4				0.7-2.4	1.4-2.4				
	OP	2 (3.0)										
Range in virus titre		2.7-3.4										
4 reference (6.1)	C	1 (1.4)	1 (2.4)									
	OP	11 (2.2)										
Range in virus titre		1.4-2.7										
5 reference (7.8)	OP	1 (1.4)										
5 reference (8.8)	C	42 (1.4)	5 (1.5)									
Range in virus titre		0.7-3.9	1.1-2.4									
	OP	6 (2.5)										
Range in virus titre		0.7-4.4										
6 reference (8.1)	C	106 (1.6)	11 (1.6)					1 (0.7)				
Range in virus titre		0.7-3.7	0.7-2.9									
	OP	12 (2.2)										
Range in virus titre		1.3-3.4										
7 reference (6.1)	C	24 (0.9)										
Range in virus titre		0.7-2.7										
	OP	21 (2.2)										
Range in virus titre		0.7-3.9										
8 reference (5.8)	C	32 (0.9)	1 (0.7)									
Range in virus titre		0.7-2.4										
	OP	7 (2.0)					1 (2.9)					
Range in virus titre		1.4-2.4										
9 32/04 (6.2)	C		1 (1.4)									
	OP	4 (2.8)										
Range in virus titre		2.4—3.4										
9 reference (7.8)	C	38 (0.8)	4 (0.9)	2 (0.7)		2 (0.9)	4 (0.9)	3 (0.7)		1 (2.4)		1 (0.7)
Range in virus titre		0.7-1.6	0.7-1.4			0.7-1.1	0.7-1.4					
	OP	1 (1.4)										

[1]$Log_{10}TCID_{50}$/mL of blood, [2] Origin of *Culicoides,* [3]Number of midges from which virus could be isolated after incubation, [4]Average virus titre/midge $log_{10}TCID_{50}$/midge, [5]Clarens, [6]Onderstepoort

Three of the ASHV isolates (the field isolates of AHSV-2 and -7 and the reference strain of AHSV-7) could not be isolated from *C. bolitinos* collected at Clarens (Table 4.12). The highest virus recovery (16.7%) in *C. bolitinos* was that of the reference strain of AHSV-3 (Table 4.12). The virus recovery of AHSV-3 was significantly higher than that of the reference strain of AHSV-8 (P=0.046) and the field isolate of AHSV-9 32/04 (P=0.016). It was, however, not significantly different from that of the references strains of AHSV-2 (P=0.696), AHSV-4 (P=0.303), AHSV-5 (P=0.383) and AHSV-9 (P=0.111).

The virus recovery rates of the reference strains of AHS-5, AHSV-6 (P<0.001 in both cases) and AHSV-7 (P=0.001) was significantly lower from *C. bolitinos* from Clarens than in *C. imicola* from Clarens. For five of the reference strains of ASHV, AHSV-1 (P=1.000), AHSV-2 (P=0.250), AHSV-3 (P=0.529), AHSV-4 (P=0.271) and AHSV-8 (P=0.070) and the field isolate of AHSV-9 (P=1.000) there were no significant difference in the virus recovery rate between *C. imicola* and *C. bolitinos* populations sampled at Clarens. For seven of the AHS strains, AHSV-1, (P=0.348), AHSV-4 (P=0.691), AHSV-5 (P=1.000), AHSV-6 (P=0.508), AHSV-8 (P=0.439), AHSV-9 (P=1.000) and the field isolate of AHSV-9 (P=0.660), the virus recovery from *C. bolitinos* from Clarens was not significantly different from that of *C. imicola* from Onderstepoort. The virus recovery of the reference strains of AHSV-2 (P<0.001) and AHSV-3 (P=0.011) was higher from *C. bolitinos* and for the reference strain of AHSV-7 it was significantly lower (P=0.024). In contrast to results with the various isolates of BTV used, the *C. bolitinos* population at Clarens did not show to be more susceptible to oral infection with AHSV than *C. imicola* collected at either Clarens or Onderstepoort. The lower virus recovery rate of AHSV compared to that of BTV in *C. bolitinos* lowers the possibility that the high recovery of BTV in this bovine dung breeder is the result of a leaky gut

(Mellor & Boorman 1980) caused by possible co-infection of this species by various micro organisms due to its breeding habitat.

The virus concentration in infected *C. bolitinos* varied from 0.7 to 2.9 $\log_{10}TCID_{50}$/midge indicating virus replication and transmission to susceptible horses may be possible (Table 4.13).

In addition to the recovery from *C. imicola* and *C. bolitinos* the reference strain of AHSV-2 was isolated from a third *Avaritia* species, namely *C. gulbenkiani* (Table 4.12). Non-*Avaritia* species from which one or more of the AHSV isolates could be isolated after incubation include *C. engubandei, C. zuluensis, C. leucostictus, C. magnus, C. enderleini, C. pycnostictus, C. exspectator* and *C. brucei* (Table 4.12). Most of these species were found widely distributed in South Africa and their host preferences included horses (Meiswinkel 2004c). Relatively high virus titre (>2.5 $\log_{10}TCID_{50}$/midge) in individual midges assayed indicate virus replication and that transmission to susceptible horses may be possible (Table 4.13).

Five of the AHSV isolates were recovered from *C. leucostictus* after incubation (Table 4.12) and relative high virus titres (>2.5 $\log_{10}TCID_{50}$/midge) in the infected individuals (Table 4.13) indicate virus replication and that transmission to susceptible horses might be possible. Relative high virus recovery rates were obtained from *C. leucostictus* and for the reference strain AHSV-2 the virus recovery rate of 38.0% was significantly higher (P=0.001) than that of *C. bolitinos.* Although *C. leucostictus* is abundant and widely distributed in South Africa, it is considered to be a bird feeder (Nevill & Anderson 1972; Nevill *et al.* 1992b; Meiswinkel *et al.* 2004c). The epidemiological significance of this finding should be further elucidated.

No virus could be isolated after incubation from a total of 152 *Culicoides* representing an additional 10 *Culicoides* species (Table 4.14). For most of these species, however, very limited numbers were tested and therefore these results are not conclusive (Table 4.14).

In contrast to previously published results, indicating AHSV to be exclusively vectored by *C. imicola,* present oral susceptibility results demonstrated that AHSV could persist for at least 10 days in nine *Culicoides* species, represented by at least six different subgenera. Cumulative oral susceptibility and field infection results indicate that susceptibility to AHSV is restricted to certain *Culicoides* species. However, this characteristic is widespread in the genus *Culicoides* and not restricted to the subgenus *Avaritia*. Considering the unique biology of vector competent *Culicoides* species one has to appreciate the complex epidemiology of orbiviruses.

Table 4.14 *Culicoides* species from which no virus could be isolated after feeding on blood containing African horse sickness virus (AHSV) after 10 days extrinsic incubation at 23.5°C

AHSV serotype and isolate	1 Reference		2 4/04		2 reference	3 Reference	4 Reference		5 reference	5 reference	6 reference	7 2/04		7 reference	8 reference		9 32/04		9 reference
Virus titre in blood meal ($log_{10}TCID_{50}$/mL of blood)	6.1		5.4		5.8	6.3	6.1		7.8	8.8	8.1	6.2		6.1	5.8		6.2		7.8
Origin	C[1]	OP[2]	C	OP	C	C	C	OP	C	C	C	C	OP	OP	C	OP	C	OP	C
Culicoides species																			
C. dutoiti	3		1														2		
C. bedfordi	4		9		1		1					3					56		
C. huambensis	1		1						1	4					3				5
C. milnei	1					1				1							1		1
C. nivosus	1				2	1				1				1				1	
C. onderstepoortensis	2				3							3					3		
C. enderleini		4						7			1					3	1		
C. subschultzei			1		1						1	2	1						
C. cornutus												1							
C. coarctatus			2		2	1						2			1		2		1

[1]Clarens, [2]Onderstepoort

CHAPTER 5

DETERMINATION OF POTENTIAL VECTORS AND FIELD INFECTION PREVALENCES IN *CULICOIDES* SPECIES IN THE WINTER RAINFALL AREA DURING OUTBREAKS OF AFRICAN HORSE SICKNESS[9]

5.1 Introduction

For at least thirty years, AHS occurred every summer in the northern provinces of South Africa, approximately once every five to ten years in the center of the country, and only rarely in the Western Cape (Bosman *et al.* 1995). Since 1950 confirmed outbreaks of AHS in the Western Cape were reported in 1967, 1990, 1999 and 2004 (Bosman *et al.* 1995; Du Plessis *et al.* 1991; Bell 1999). Each time it has been postulated that outbreaks of AHS in the Western Cape resulted from the transportation of viraemic horses from endemic areas (Bosman *et al.* 1995; Bell 1999; Guthrie 1999).

In a six year survey (1979 to 1985), *Culicoides* midges were collected at 25 sites throughout South Africa, and 918 virus isolates were made from 4 506 pools of midges (Nevill *et al.* 1992a). While AHSV was isolated in 66 cases, of which 94% were from midges collected in the northern regions of the country, it was not isolated from midges collected in the Western Cape (Nevill *et al.* 1992a). Prior to 1999 only two light trap surveys were conducted in the Stellenbosch area (Venter *et al.* 1987; Nevill *et al.* 1988; Venter *et al.* 1996b; 1997). As part of a countrywide survey (Nevill *et al.* 1992b; Venter *et al.* 1996b; 1997) 62 collections were made from January 1984 to November 1986 at one site (near cattle on the Welgevallen Research Farm) and in November 1986 a more intensive survey was conducted at 22 sites in the Stellenbosch

[9] Partially published as:

Venter, G.J., Koekemoer, J.J.O. & Paweska, J.T. (2006) Investigations on outbreaks of African horse sickness in the surveillance zone of South Africa. *Revue scientifique et technique, Office International des Epizooties,* **25,** 1 097-1 019.

area (Nevill *et al.* 1988). These surveys indicated that *C. imicola*, was less abundant than in the traditional AHS endemic summer rainfall areas of the country. The November 1986 Stellenbosch survey (Nevill *et al.* 1988) confirmed the results of the two year Welgevallen survey (1984-86) (Venter *et al.* 1987) which revealed that *C. gulbenkiani, C. magnus, C. zuluensis* and *C. imicola* alternated as the dominant species in light trap collections. Data from the two year study indicated that *C. imicola* constituted 11.3% of 145 297 midges collected in 62 collections (the average being 265 specimens per trap per night) (Venter *et al.* 1996b; 1997). In the 1986 study *C. imicola* represented 18.4% of 33 564 *Culicoides* collected in 44 collections (the average numbers of *C. imicola* collected per night being 141 specimens) (Nevill *et al.* 1988). This relatively low abundance of *C. imicola* was in contradiction to the rest of the country, and especially the traditional AHS endemic areas, where *C. imicola* can become superabundant near livestock (Mellor *et al.* 2000; Meiswinkel *et al.* 2004c). For example, *C. imicola* represented 98.0% and 93.0% of 104 720 midges (146 collections) and 1 730 191 midges (39 collections) respectively, collected at the ARC-OVI (Venter *et al.* 1996b) and the Eiland holiday resort both in the northern regions of the country (Venter *et al.* 1997).

Outbreaks of AHS in 1999 and again in 2004 in the Stellenbosch area, which constitutes the surveillance zone of the declared AHS free area (Bosman *et al.* 1995; Guthrie 1999), has each time led to a two year embargo on the export of horses from South Africa with a loss of about R50 million (USD8.2 million) annually. During the 1999 outbreak of AHS, 14 deaths had been reported during a period of one week on seven premises as a result of infection with AHSV-7 (Bell 1999). In the 2004 outbreak, 16 deaths on eight premises and 16 clinical cases on a further 11 premises were reported from February to April 2004, as a result of infection with AHSV-1. Because the Stellenbosch area falls within the surveillance zone in the Western Cape, none of the 8 000 equines in this area were vaccinated against AHS. However, in an

effort to contain the spread of this virus during each of the last two outbreaks all equines in this area were vaccinated with the commercially available polyvalent AHS vaccine. The 1999 and 2004 outbreaks of AHS in the Stellenbosch area prompted a further entomological and virological investigation, which results are presented in this chapter.

5.2 Material and Methods

5.2.1 Study area

The "Stellenbosch area" referred to in the current study is similar to the area surveyed in 1986 (Nevill *et al.* 1988) and covered 540 km^2 from 33˚40'-34˚00'S and from 18˚40'-19˚00'E. The south-eastern part is mountainous, with wide, flat valleys open to the north-west into gently rolling countryside. This is a winter rainfall region receiving from 600 to 1 000mm per annum, depending on the proximity to the mountains where precipitation is higher (South African Weather Bureau 1986). The six months from April to September receive 75% of the annual total rainfall for the region. Summers are relatively hot. This is a virtually frost-free area so temperatures below freezing and frost are seldom recorded. At Elsenburg the monthly mean daily minimum for the coldest month (July) was 7.0 °C (South African Weather Bureau 1986). The natural vegetation of the study area is classified as Coastal Rhenoster-bosveld and Macchie or Fynbos (Acocks 1975), however, except in protected areas, very little of this vegetation remains. Much of the region is intensively farmed, especially where water is available for irrigation during the dry summer. Here the production of grapes and deciduous fruits is important, as are kikuyu, white clover and other pasture types for intensive livestock farming. Where less water is available, winter wheat is planted and livestock are raised on natural grazing (Nevill *et al.* 1988).

5.2.2 Sampling of *Culicoides* midges for determining field infection prevalence

In order to assess the possibility of midges having been infected with AHSV in the field, parous *C. imicola* and *C. bolitinos* females were collected directly into phosphate buffered saline (PBS) to which 0.5% 'Savlon' antiseptic was added (Walker & Boreham 1976b). After retrieval in the morning, the collections were kept at 4°C until processed for the presence of virus. During species identification, *Culicoides* were age-graded according to the method of Dyce (1969), and sorted in PBS into pools of mostly 100 to 500 parous females per species. As there is no proof that orbiviruses can be transmitted transovarially in *Culicoides* species (Jones & Foster 1971; Nelson & Scrivani 1972; Nunamaker *et al.* 1990) only parous females were used in virus isolation attempts.

5.2.3 Virus isolation from midge pools

Midge pools were washed twice in fresh PBS and then homogenized in chilled 250 µl EMEM containing antibiotics (200 µg/mL of penicillin/streptomycin and 2.5 µg/mL of fungizone) using a battery-powered microtissue grinder (Venter *et al.* 1998). Homogenized samples were centrifuged at 14 000 x g for 10 minutes at 4 °C and the individual supernatants transferred into sterile 1.5 mL microtubes. The pellet was disrupted by the freeze-thaw technique and homogenized in 100 µl of EMEM. Homogenized pellet samples were centrifuged at 14 000 x g for 10 minutes at 4 °C and the individual supernatants added to the first supernatant to which 1 650 µl of EMEM was added. Samples were then filtered using 0.2 µm filters and 20% foetal-calf serum was added. A half volume (1 mL) of each individual homogenate was diluted 1:2 in EMEM, and then 250 µl of this dilution inoculated into two 25 cm^2 plastic culture flasks containing 48 hour-old monolayers of Vero cells. After an hour of incubation at 37 °C on a platform shaker, inoculated cells were rinsed once with sterile PBS, overlaid with 10mL EMEM containing 1% foetal-calf serum, and then

incubated at 37 °C in an incubator containing 5% CO_2. Inoculated cells were examined microscopically daily for 10 days post inoculation for CPE. In the absence of CPE, cultures were frozen at -70 °C, thawed, and re-passaged for two blind passages (Meiswinkel & Paweska 2003).

5.2.4 Oral susceptibility

High abundance of especially *C. imicola* rendered the horse stable at Linquenda (33°59.2S, 18°48.82E; 20 m above sea level) on the outskirts of Stellenbosch as highly suitable for vector competence studies. *Culicoides* were collected in January and again in April 2006. Four to five light traps were used to capture *Culicoides* species alive at Linquenda. Midges were couriered to the ARC-OVI. Four of the most recent AHSV isolates in South Africa were obtained from the OIE Reference Centre for African horse sickness at the ARC-OVI. Field viruses were isolated and identified as described for the field isolates of AHSV-7. The geographical origin of the field isolates of AHSV-7 used is shown in Table 5.1. Artificial infection, incubation and virus isolation from the incubated midges were done as described in Chapter 4.

Table 5.1 Designation and origin of African horse sickness virus serotype 7 (AHSV-7) field isolates used for comparative oral susceptibility studies

AHSV serotype (isolate designation)	Year of isolation	Locality
3 (67/04)	2004	Stellenbosch
4 (70/04)	2004	Botswana
6 (1/04)	2004	Vryheid
7 (57/04)	2004	Senekal

5.3 Results and Discussion

5.3.1 Light trap surveys

During the 1999 survey a total of 212 199 *Culicoides* midges, belonging to 13 species, was collected from 26 March to 29 April 1999 in 38 light trap collections made at 15 sites. Depending on the prevailing weather conditions on the night of collection, the number of hosts present and the location of the light trap, the number of *Culicoides* midges collected per light trap per night ranged from 5 to 75 116 (average = 5 584) (Table 5.2). *Culicoides imicola* represented 96.0% of the total number of midges collected and was the dominant *Culicoide*s species at all 15 sites sampled (Table 5.2). *Culicoides imicola* and *C. magnus* (representing 0.7% of all *Culicoides* species collected), were the only two species to be present at all 15 collection sites. The second most abundant species, *C. zuluensis,* was collected at 14 of the 15 sites sampled and represented 1.7% of all the *Culicoides* collected. *Culicoides bolitinos* was found at 13 of the 15 sites sampled and was the third most abundant species representing 1.1% of all *Culicoides* collected. A further 10 *Culicoides* species, each representing less than 1%, were collected (Table 5.2). During the 2004 survey a total of 187 522 *Culicoides* midges, belonging to 15 *Culicoides* species, were collected in 39 light trap collections made from 1 to 26 March 2004 at 16 sites. The number of midges collected per trap per night ranged from 1 to 63 260 (average = 4 808) (Table 5.3). Similar to the 1999 survey, *C. imicola,* representing 94.6% of all *Culicoides* collected, was shown to be the dominant species at all sites. It was also the most widespread as it was the only *Culicoides* species to be present at all of the sites sampled. As in the 1999 survey the second and third most dominant species to be collected were *C. zuluensis* (3.1%) and *C. bolitinos* (1.4%), respectively. In the 2004 survey, *C. zuluensis* and *C. bolitinos* were collected at 14 and 13 of the 16 sites sampled, respectively. A further 12 *Culicoides* species, each representing less than 1%, were collected (Table 5.3).

Culicoides magnus and *C. gulbenkiani,* shown to be the co-dominant species in 1986 (Nevill *et el.,* 1988), represented less than 1% of all *Culicoides* species collected in both the 1999 and 2004 surveys (Table 5.4). In all three surveys *C. imicola* was found to be widely distributed in the area, as it was present at all sites sampled. However, in the 1999 and 2004 surveys it was the dominant species at all of the sites sampled which is significantly different ($P<0.001$) from the 1986 survey, where it was dominant at five of the 22 sites sampled (Table 5.4). While there was no significant difference ($P>0.05$) in the average number of *C. imicola* collected per night at each site between the 1999 and 2004 surveys, significantly lower numbers of *C. imicola* were collected per site in the 1986 survey than in either of the 1999 ($P<0.001$) or 2004 ($P<0.05$) surveys.

Similarly there were no significant differences ($P>0.05$) in either of the average numbers of *C. magnus* or *C. pycnostictus* collected per night at each site in the 1999 and 2004 surveys. Significantly higher numbers of *C. magnus* were, however, collected per site in the 1986 survey than in either of the 1999 ($P<0.001$) or 2004 ($P<0.01$) surveys. In addition, significantly higher numbers of *C. pycnostictus* were collected per site in the 1986 survey than in either of the 1999 ($P<0.001$) or 2004 ($P<0.001$) surveys.

The average number of *C. bolitinos* ($P=0.275$) and *C. zuluensis* ($P=0.237$) collected per night at each of the sites did not differ significantly between the three surveys. *Culicoides bolitinos* was collected at all 22 sites sampled in 1986, and was the dominant species at two of these sites. This was, however, not significantly different from either the 1999 survey ($P=0.158$) or the 2004 survey ($P=0.066$), when it was collected at 13 of 15 and 16 sites, respectively.

Table 5.2 Identification of the *Culicoides* species recovered from black light traps during an outbreak of AHS in the Stellenbosch area during March to April 1999 indicated as percentages

Collection site	Linquenda	Thistledown	Stellen Kloof	Remhoogte	Spier	Avontuur	Varswater	Maties Ryskool	Trough End	Elsenburg	Klein Bosch	Sandringham	Rosendal	Digteby	Glenconner
Collection date	27/3-20/4	22/4	29/3-14/4	26/3	30/3-21/4	3-30/4	23/4	28-30/3	2/4	22/4	15/4	24/4	26/3-14/4	31/3	29/4
No of collections made	3	1	2	1	4	4	1	2	1	1	1	1	14	1	1
Culicoides species															
C. imicola	98.52	97.96	97.01	84.88	96.67	89.28	96.93	97.62	99.02	92.29	99.50	84.00	44.17	97.5	43.24
C. zuluensis	0.37	1.84	1.00	7.16	1.23	6.11	1.65	0.02	0.20	2.57	0.45	12.00	17.57		5.41
C. bolitinos	1.08	0.07	0.40	0.75	1.76	1.00	0.11	2.10	0.49	3.04		1.71	0.25	2.5	
C. magnus	<0.01	0.07	0.10	7.11	0.32	3.37			0.29	0.76		1.71	2.74		
C. leucostictus	<0.01		1.10			0.04	1.31	0.13					23.93		32.43
C. pycnostictus			0.30			0.03					0.06	0.29	8.22		18.92
C. gulbenkiani	0.02	0.07	0.02	0.05	0.02	0.03		0.13		1.33		0.29	0.12		
C. nivosus				0.05		0.03							2.31		
C. nr angolensis	<0.01		0.07			0.06							0.44		
C. exspectator						0.04									
C. engubandei													0.12		
C. coarctatus													0.06		
C. sp.107													0.06		
Total	107 913	10 633	13 667	8 044	25 402	24 506	4 429	8 614	3 066	2 102	1 791	350	1 605	40	37
Average catch size	35 971	10 633	6 833.5	8 044	63 50.5	6 126.5	4 429	4307	3 066	2 102	1791	350	114.61	40	37
Range in catch size	2 157 to 75 116	10 633	5 557 to 8 110	8 044	294 to 21 740	58 to 11 540	4 429	654 to 7 960	3 066	2 102	1791	350	5 to 358	40	37

Table 5.3 Identification of the *Culicoides* species recovered from black light traps during an outbreak of AHS in the Stellenbosch area during March 2004 indicated as percentages

Collection site	Linquenda	Spier	Rosendal	Natte Valeij	Del Vera	Elsenburg	Vredenheim	Dakari	Dalewood	Langeberg	Chilanga	Paarl Diamond	Delmerdo (Ashire)	Through End	Ivan Stark	Staedfast Farm
Collection date	1/3	1-26/3	2/3	3/3	3-30/3	1-26/3	18-19/3	1/3	2/3	3/3	11-15/3	4/3	4/3	2/3	4/3	4/3
No of collections made	1	5	1	1	6	10	2	1	1	1	5	1	1	1	1	1
Culicoides species																
C. imicola	99.07	99.22	75.49	96.33	98.44	77.61	45.61	79.44	54.99	94.68	79.23	92.31	91.43	94.44	61.54	100
C. zuluensis	0.48	0.29	17.76	2.01	0.52	13.11	43.06	12.07	41.71	2.88	0.24	3.85	5.71	5.56		
C. bolitinos	0.38	0.27	3.10	0.45	0.78	8.42	6.74	1.16	0.21	0.78	0.35	1.92			30.77	
C. magnus	0.02	0.13	3.60	0.80	0.14	0.64	1.43	1.74	2.99	1.11			2.86			
C. leucostictus		0.02		0.15	0.01	0.02	1.18			0.11	16.86					
C. gulbenkiani	0.02					0.17	1.04	5.31								
C. pycnostictus			0.05	0.19		0.03	0.07			0.33	2.89	1.92			7.69	
C. glabripennis	0.02	0.08														
C. nivosus					0.07	0.01	0.82		0.10		0.17					
C. onderstepoortensis				0.08				0.29			0.03					
C. milnei	0.02															
C. neavei										0.11	0.10					
C. angolensis											0.07					
C. similes							0.04									
C. engubandei											0.03					
Total	25 210	110 842	3 998	2 641	145 11	21 623	2 789	1 036	971	902	2870	52	35	18	13	11
Average catch size	25 210	22 168.4	3 998	2 641	751.8	2 162.3	1 393	1 036	971	902	574	52	35	18	13	11
Range in catch size	25 210	48 to 63 260	3 998	2 641	1 to 14 465	1 to 16 887	1 100 to 1 689	1 036	971	902	166 to 1 029	52	35	18	13	11

Table 5.4 Comparison of black light trap collections made during 1986, 1999 and 2004 in the Stellenbosch area, south-western Western Cape, South Africa.

Collection date No of sites sampled (No of collections made)	November 1986 (Nevill *et al.* 1988) 22 (44)			March - April 1999 15 (38)			March – April 2004 16 (39)		
Culicoides species	No of sites present (no of sites dominant)	Total	Average (%)	no of sites present (no of sites dominant)	Total	Average (%)	no of sites present (no of sites dominant)	Total	Average (%)
***Culicoides* species**									
C. imicola	22 (5)	6 186	140.6 (18.4)	15 (15)	203 774	5 362.5 (96.0)	16 (16)	177 455	4 550.1 (94.6)
C. zuluensis	22 (1)	4 122	93.7 (12.3)	14	3 588	94.4 (1.7)	14	5 878	150.7 (3.1)
C. bolitinos	22 (2)	5 018	114.0 (15.0)	13	2 258	59.4 (1.1)	13	2 685	68.8 (1.4)
C. magnus	22 (6)	8 376	190.4 (25.0)	15	1 576	41.5 (0.7)	11	571	14.6 (0.3)
C. leucostictus	21 (2)	3 750	85.2 (11.2)	6	627	16.5 (0.3)	7	547	14.0 (0.3)
C. gulbenkiani	19 (2)	2 793	63.5 (8.3)	3	90	2.4 (<0.1)	4	126	3.2 (0.1)
C. pycnostictus	22 (3)	1 990	45.2 (5.9)	10	190	5.0 (0.1)	8	103	2.6 (0.1)
C. glabripennis							2	97	2.5 (0.1)
C. nivosus	19 (1)	736	16.7 (2.2)	4	49	1.3 (<0.1)	5	41	1.1 (<0.1)
C. nr angolensis					33	0.9 (<0.1)			
C. onderstepoortensis	2	27	0.6 (0.1)				3	6	0.2 (<0.1)
C. exspectator	4	43	1.0 (0.1)	1	10	0.3 (<0.1))			
C. milnei							1	5	0.1 (<0.1)
C. neavei	3	6	0.1 (<0.1)				2	4	0.1 (<0.1)
C. angolensis	5	60	1.4 (0.2)				1	1	0.03 (<0.1)
C. similes	4	28	0.6 (0.1)				1	1	0.03 (<0.1)
C. engubandei				1	2	0.10 (<0.1)	1	1	0.03 (<0.1)
C. coarctatus[1]	1	3	0.1 (<0.1)	1	1	0.03 (<0.1)			
C. brucei	1	2	0.05 (<0.1)						
C. accraensis	1	10	0.2 (<0.1)						
C. olyslageri	3	12	0.3 (<0.1)						
C. nevilli	2	16	0.4 (0.1)						
C. huambensis	14	338	7.7 (1.0)						
C. subschultzei	4	21	0.50 (0.1)						
C. enderleini	1	1	0.02 (<0.1)						
C sp 107				1	1	0.03 (<0.1)			
Other	8	26	0.6 (0.1)						
TOTAL		33 564			212 199			187 522	

[1]Identified as *C. confuses* in the survey of Nevill *et al.* (1988)

5.3.2 Field infection prevalence

To determine the infection prevalence in field-collected *Culicoides* species during the 1999 outbreak of AHS, 331 pools consisting of 100 and three pools consisting of 500

parous *C. imicola* females were analyzed for the presence of virus (Table 5.5). While EEV-1 was isolated from 13 of these pools, AHSV-7 was only isolated once (Table 5.5). Field infection prevalence in *C. imicola* was calculated, using the method of Walter *et al.* (1980), to be 0.003% for AHSV and 0.038% for EEV. Based on the field infection prevalence of the 1999 survey the pool size was increased to 500 individuals in the 2004 survey. The number of pools tested and the pool sizes are given in Table 5.5. AHSV-1 was isolated from two of these pools. AHSV-7 and BTV were also each isolated from one of these pools (Table 5.5). The field infection prevalence in *C. imicola* during the 2004 outbreak was calculated to be 0.005% for AHSV-1 and 0.002% for AHSV-7 and BTV.

The fact that AHSV, BTV as well as EEV could be isolated from field-collected *C. imicola* in this study confirms that vector competent populations of this species are present in this area. Although only AHSV-1 was isolated from sick and dead horses as well as from field-collected midges during the 2004 outbreak, AHSV-7 was only isolated from field-collected *C. imicola* (Table 5.5). The 2004 results indicated that in addition to AHSV-1, which had caused deaths in horses, at least one more incursion of AHS into the Stellenbosch area had taken place during this time.

Table 5.5 Virus isolation results from midge pools collected with black light traps during the 1999 and 2004 outbreaks of AHS in the Stellenbosch area, south-western Western Cape, South Africa

Farm Name	Collection date	*Culicoides* species	Pool size	No. of pools tested	Viruses isolated
1999					
Avontuur	2/4	*C. imicola*	100	46	
"	4/4	"	"	10	4 x EEV-1
Maties ryskool	28/3	"	"	20	2 X EEV-1
Remhoogte	25/3	"	"	30	
Rosendale	25/3	"	"	1	
Spier	14/4	"	"	33	
"	29/3	"	"	49	
Stellenkloof	28/3	"	"	29	2 X EEV-1
Troughend	2/4	"	"	6	
Linquenda	27/3	"	"	107	4 X EEV-1
"	"	"	500	3	1 X EEV-1 1 X AHSV-7
TOTAL			**1 500**	**334**	
2004					
Elsenburg	2/3	*C. imicola*	105	1	
"	3/3	"	500	7	2 X AHSV-1
"	3/3	*C. bolitinos*	191	1	
"	3/3	*C. imicola*	306	1	
"	3/3	"	500	1	
"	4/3	"	135	1	
"	11/3	"	375	1	
"	11/3	*C. bolitinos*	176	1	
"	18/3	"	121	1	
Linquenda	1/3/	"	500	18	1 X BTV
Del Vera	3/3	"	500	6	
Rosendal	2/3	"	500	1	
"	2/3	"	412	1	
Dalewood	2/3	"	185	1	
Natte Valeij	3/3	"	109	1	
"	3/3	"	500	1	
Dakari	1/3	"	259	1	
Vredenheim	18/3	"	500	1	
"	19/3	*C. imicola*	172	1	
Chilanga	14/3	"	129	1	
"	18/3	"	145	1	
Spier	24/3	"	500	3	
"	24/3	"	135	1	
"	25/3	"	500	18	
"	26/3	"	500	26	1 X AHSV-7
TOTAL			**7 955**	**97**	

5.3.3 Oral susceptibility

The feeding rates for field-collected *Culicoides* females varied from 10-70%. Of 1 657 *Culicoides* fed 630 (38.0%) survived the extrinsic incubation period. All four viruses used were recovered from *C. imicola* after incubation. Relative high virus titres (>2.5 $log_{10}TCID_{50}$/midge) in individual midges assayed indicated virus replication and that transmission to susceptible horses might be possible (Table 5.6). The highest recovery rate was that of AHSV-6 (8.5%). It was, however, only significantly higher than that of AHSV-3 (P=0.001). No virus could be isolated from single specimens of *C. bolitinos* and *C. zuluensis* tested after incubation (Table 5.6).

These oral susceptibility results indicated that the *C. imicola* populations at Stellenbosch are highly susceptible to infection with at least four serotypes of AHSV. Coupled to the field isolation of AHSV from *C. imicola* these results leave no doubt that this species will very effectively transmit AHSV in the area.

Table 5.6 Virus recovery rates and titres of field-collected *Culicoides* midges maintained for 10 days at 23.5 ^{o}C after feeding blood containing African horse sickness virus

Culicoides species	*C. imicola*		*C. bolitinos*	*C. zuluensis*
AHSV serotype/ isolate (Virus titre of blood meal[1])	No pos/No test (%)	Titre[2] (range in virus titre))	No pos/No test (%)	No pos/No test (%)
3 67/04 (5.7)	1/149 (2.0)			2
4 70/04 (6.0)	7/203 (3.4)	1.2 (0.7-2.4)	1	
6 1/04 (6.1)	12/142 (8.5)	2.1 (0.7-2.9)		
7 57/04 (6.1)	3/70 (4.3)	0.7		

[1]$Log_{10}TCID_{50}$/mL of blood, [2]Average virus titre $log_{10}TCID_{50}$/midge

Incursions of AHSV can be either via the movement of viraemic horses from infected zones or via infected midges. Except for a single mountain range the borders

of the various zoning regimes are mostly administrative and do not safeguard this area from the movement of *Culicoides* midges, especially along the coast. There is compelling evidence that *Culicoides* midges can be transported by prevailing winds for hundreds of kilometres (Sellers *et al.* 1977). It is, however, also possible that they can disperse with livestock (Meiswinkel *et al.* 2004c). Since little is known about reservoir hosts in the surveillance zone and adjoining areas, it is not possible to rule out the possibility that infected midges will enter or be present in these areas. Considering these uncertainties, together with the relatively high abundance of *Culicoides* species in the surveillance zone it is advisable that susceptible horses be protected against AHS at all times.

Taking into account the number of mammals in the area in relation to the relatively low number of viraemic horses, the low infection prevalence (0.003% for AHSV and 0.038% for EEV) detected in field-collected midges is typical of an arbovirus outbreak (Chiang & Reeves 1962; Walter *et al.* 1980). However, the superabundance of *C. imicola*, as determined by light trap collections, compensate for the relatively low infection prevalence.

It was found that numbers of *Culicoides* species and especially *C. imicola* collected during the 1999/2004 surveys could exceed those in the traditional AHS endemic areas during the same period. Since there is no evidence that AHSV is transmitted transovarially by the vectors, persistence is only possible in those areas where active adult vectors are present throughout the year. For example, *C. imicola* can be collected in low numbers throughout winter at Onderstepoort which is an area which experiences frost (Venter *et al.* 1997). Stellenbosch is a frost-free winter rainfall area so it is likely that *Culicoides* adults will also be present in this area in winter (Venter *et al.* 1997). If a suitable reservoir host for AHSV is present, it will therefore be possible for AHSV to overwinter in the Stellenbosch area. To ensure that the transmission of AHSV is successfully broken, vector-free periods must be of longer

duration than the maximum period of viraemia in the local susceptible vertebrate population (Mellor 1994). In this context, it is worthwhile to mention recent work showing that it is possible to detect BTV nucleic acid by nested RT- PCR in *C. sonorensis* larvae (White *et al.* 2005). The published data also suggested that BTV may not require abundant expression of the outer coat genes to persist in the insect vector and that this could explain the low rate of isolation of virus from insects (White *et al.* 2005).

The results of the March/April 1999/2004 surveys on *Culicoides* species composition and abundance, indicated that the proven AHSV vector, *C. imicola,* is up to 400 times more abundant than was shown by either the November 1986 (Nevill *et al.* 1988) survey or the two year survey conducted from 1984 to 1986 (Venter *et al.* 1997). Because of seasonal variation and variation in climatic conditions between years, together with environmental factors on the night of collection at each site, direct comparison between these datasets is not entirely possible and conclusions must be made cautiously. It can, however, be pointed out that the maximum catch size for *C. imicola* during the entire two year Welgevallen survey (1984-86) was 5 540 specimens (representing only 36.6% of more than 15 000 midges) collected in April 1986. In contradiction to the April 1986 data, *C. imicola* was collected in very low numbers in April 1985 (5.6% of 1 632 midges) and April 1984 (2.5% of 1 997 midges) at Welgevallen. The maximum number of *C. imicola* collected during the November 1986 survey (Nevill *et al.* 1988), were 1 123 specimens, representing 60.3% out of a total of 1 863 midges collected.

During the 1999/2004 surveys *C. imicola* was, as in the traditional AHS endemic areas, superabundant and the most dominant *Culicoides* species in the area. It would, therefore play an important role in the transmission of AHSV in this area. Serendipitously this apparent increase in numbers in comparison to previously published data for the Stellenbosch area coincided with the northwards expansion of

C. imicola into many areas of Europe, attributed to climate change (Purse *et al.* 2005). The closely related BTV, also transmitted by *C. imicola,* has already entered many parts of Europe and is causing unprecedented outbreaks of disease in ruminants, suggesting that AHSV could do the same in equids (Mellor & Hamblin 2004). The abundance of most of the other *Culicoides* species collected in the present study did not change significantly from previous collections made in the area. To determine if the high numbers of *C. imicola* collected in the 1999/2004 surveys is a true reflection of the status of this vector or just a coincidence of yet undefined factors and to confirm the absence of vector free periods it will be necessary to conduct a more intensive and seasonal *Culicoides* survey in this area.

Entomological and virological results presented for the Stellenbosch area in this study demonstrated that the designation of the AHS-free zone in the southwest of the Western Cape remains highly controversial. The veterinary policy of maintaining large populations of unvaccinated horses in the surveillance zone is of concern since it leaves horses vulnerable to infection with the most deadly virus of equids and, as evidenced by the 1999 and 2004 outbreaks, this zone can easily be compromised. It is, therefore, postulated that the maintenance of the zoning system for AHS, as in the present veterinary policies and its geographical boundaries, be reconsidered.

CONCLUSIONS

1 Black light is more effective than white light for attraction and collection of *C. imicola* and other livestock-associated South African *Culicoides* species.

2 Infection rates in *C. imicola* and *C. bolitinos* as determined with membrane feeding are higher compared to cotton wool feeding but the latter makes it possible to distinguish the levels of competence between *Culicoides* species for orbiviruses. Reduced infection rates with cotton pledget feeding are partly due to a smaller volume of blood meals taken. The pledget method will be useful when attempting to assess the competence of field-collected vectors that are reluctant to blood feeding via a membrane or on the natural host.

3 The average blood meal size for *C. imicola* as determined by membrane feeding is 0.045 µl. The implication is that the minimum virus titre in the blood should be at least $10^5 \log_{10}$ID/mL of blood to ensure that each midge will theoretically take up at least one virus infection dose on feeding.

4 *Culicoides bolitinos* is significantly more susceptible to oral infection with most of the BTV strains than *C. imicola*.

5 Bluetongue virus and AHSV replicate in *Culicoides* species of six different subgenera thereby implicating multi-vector potential for the transmission of these viruses.

6 Laboratory attenuation of BTV and AHSV does not reduce their ability to infect competent *Culicoides* species and may even lead to enhanced replication in the vector.

7 Differences found in the oral susceptibility of *C. imicola* and C. *bolitinos* for isolates of the same serotypes of AHSV, suggest co-adaptation between orbiviruses and vectors present in a given locality. Real-time monitoring of

vector competence might be difficult as it would require assessing local *Culicoides* populations using variants of orbiviruses currently in circulation.

8 *Culicoides imicola* was shown to be, as in the summer rainfall region, a dominant species in the winter rainfall region in South Africa and capable of replicating viruses belonging to different serogroups of the *Orbivirus* genus.

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OPSOMMING

Culicoides (Diptera, Ceratopogonidae) bytende muggies speel ‘n onontbeerlike rol in die oordraging van orbivirusse. Die huidige uitbrake van bloutong (BT) in Europa illustreer die gevaar vir die invoering en vinnige verspreiding van vektor oorgedraagde siektes buite hul gevestigde voorkomsgebiede en het gelei tot verhoogde internasionale belangstelling in die epidemiologie van arbovirusse. Die siektes wat deur perdesiektevirus (PSV) en bloutongvirus (BTV) veroorsaak word, het ‘n hoë sosio-ekonomiese impak, veral met betrekking tot internasionale handel. Die identifisering van moontlikke vektore is ‘n belangrike voorvereiste vir die implementering van intergrale beheermaatreëls, risikobepaling en siektebestuur. Die bepaling van die mondelingse vatbaarheid van *Culicoides* spesies vir besmetting met orbivirusse gee waardevolle inligting vir die bepaling van die vektorbevoegdheid van spesies.

Die doel van die studie was om die mondelingse vatbaarheid van muggie spesies, wat by plaasdiere aangetref word, vir veld- en lewend geatenueerde entstofstamme van BTV en PSV te bepaal. ‘n Verdere doel was om die geskiktheid van verskillende ligbronne vir die versameling van muggies te evalueer en om kunsmatige bloedvoedingmetodes te vergelyk. Die identifikasie van potensiële vektore en die bepaling van veldinfeksie in *Culicoides* spesies tydens uitbrake van PS in die winterreënvalgebied van Suid-Afrika is ook gedoen.

Alhoewel daar geen verskil in die ouderdomsamestelling van *Culicoides* bevolkings soos met swart en witlig versamel is nie, was swartlig meer effektief vir die versameling van volwasse *Culicoides* muggies. Infeksiesyfers soos bepaal met kunsmatige voeding op bloed deurdrenkte watte was laer as die bepaal met membraamvoeding. Die verskil is die gevolg van ‘n groter bloedmaal wat deur *Culicoides* wyfies opgeneem word tydens membraanvoeding. Die isolasie van beide

die verwysing- en die entstofstamme van BTV was hoër in *C. bolitinos* as in *C. imicola.* Daar was egter geen betekenisvolle verskil in die vatbaarheid van *C. bolitinos* en *C. imicola* vir die verskillende PSV isolate wat gebruik is nie. Die isolasie van die entstofstam van PSV-7 uit *C. imicola* wat by Onderstepoort versamel is, was hoër as die van die veldisolate van PSV-7. *Culicoides imicola* wat in die oos Vrystaat versamel is, was meer vatbaar vir PSV as *C. imicola* wat in Gauteng versamel is. Beide BTV en PSV was geïsoleer uit *Culicoides* spesies wat nie aan die subgenus *Aviritia* behoort nie. Die resultate dui daarop dat BTV en PSV deur meer as een vektor oorgedra kan word en verhoog dus die kompleksiteit van die epidemiologie van die siektes. Dit sal relatief moeilik wees om die ware bepaling van die vektorbevoegdheid van ‘n spesie te doen, aangesien dit onder andere die toetsing van plaaslike *Culicoides* bevolkings met die virusstam wat die uitbraak veroorsaak sal behels.

Ligvalopnames tydens uitbrake van PS in die wintereënvalgebied van Suid-Afrika het aangetoon dat *C. imica* baie volop is en dat die getalle wat versamel kan word gelyk of selfs hoër kan wees as die getalle wat in die somerreënvalgebiede versamel kan word. Resultate van vatbaarheid en die isolasie van veldstamme van verskeie spesies van orbivirusse dui daarop dat die *C. imicola* bevolking in die winterreënvalgebied ‘n hoogs bekwame en doeltreffende vektor van orbivirusse is.

ADDENDUM 1

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ADDENDUM 2

Reprints of articles originating from this study

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 142 (2006) 383–385
veterinary parasitology
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Short communication

Comparison of black and white light for collecting *Culicoides imicola* and other livestock-associated *Culicoides* species in South Africa

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Received 3 May 2006; received in revised form 7 June 2006; accepted 18 July 2006

Abstract

Comparison of the effectiveness of 8 W fluorescent black and white light sources, in two 4 × 4 Latin squares (16 replicates) designs under South African conditions, showed black light to be up to three time more effective in collecting *Culicoides imicola* Kieffer (Diptera, Ceratopogonidae) and other South African *Culicoides* species. Four *Culicoides* species, which were collected in low numbers with black light, were not collected in traps equipped with the white light source. No significant difference was found in the parous rate of the *C. imicola* populations as determined by the two light sources. The study highlighted the superiority of black light as a preferred collection method for *C. imicola*, considered to be the most widespread and abundant vector of livestock orbiviruses. The results underline the need to develop and adopt standard techniques for measuring the variables of vectorial capacity.

Keywords: Black light; *Culicoides imicola*; South Africa

1. Light source comparisons

Several species of *Culicoides* biting midges (Diptera, Ceratopogonidae) have been shown to be involved in the transmission of a variety of pathogens, the most important being the viruses that cause bluetongue and African horse sickness. The primary monitoring tools used to determine the presence and seasonal abundance of these vectors are various designs of light traps.

It is known that black light is more effective than white light for the collection of night-flying insects in general and also for *Culicoides*, especially *C. sonorensis* Wirth and Jones, the principle vector of bluetongue in North America (Anderson and Linhares, 1989; Wieser-Schimpf et al., 1990). *Culicoides* are, however, a biologically highly diverse genus, with more than 1219 described species (Meiswinkel et al., 2004), and the extrapolation of data from one species to others from different geographical localities is not recommended. No data are available on the differential attraction of white and black light for South African *Culicoides* species, especially *Culicoides imicola* Kieffer, considered to be one of the most widespread and abundant vectors of livestock orbiviruses (Purse et al., 2005).

To determine the differential attraction of white and black light on the numbers of *Culicoides* collected, species composition and parous rates under South African conditions, a study was conducted at ARC-Onderstepoort Veterinary Institute (ARC-OVI). This

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0304-4017/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2006.07.015

involved the use of four Onderstepoort suction light traps (Venter and Meiswinkel, 1994), two equipped with 8 W 23 cm black lights and two equipped with 8 W 23 cm white lights, compared in two replicates of a 4 × 4 randomized Latin square design (Snedecor and Cochran, 1980). Suction was provided by panel fans with an average air displacement capacity of 204.5 m^3/min (S.D. = 9.47). Light traps were operated 10–15 m apart at opposite ends of a stable housing 15–20 horses. Trapping was conducted from dusk to dawn on 10 consecutive nights in the early part of summer from 26 October to 4 November 2004. Due to adverse weather conditions on two nights treatments had to be repeated the following nights. The females of all species were age-graded into nulliparous, parous, gravid females or freshly bloodfed females. Males and all other insects captured were also counted.

Significantly more ($P < 0.001$, $F = 16.71$, d.f. = 1) *Culicoides* were collected with traps equipped with a black light source (Table 1). Four *Culicoides* species, which were collected in very low numbers with black light, were not collected with white light (Table 1). Of the 22 *Culicoides* species collected, 19 were present in greater numbers in traps equipped with black light sources.

C. imicola was the dominant species in both the black and white light trap collections but significantly more *C. imicola* ($P < 0.001$; $F = 16.35$, d.f. = 1) were collected with the black light than with white light (Table 1). Contrary, however, to results for *C. sonorensis*, where black light was shown to be 11 times more efficient than white light (Rowley and Jorgensen, 1967), the present study indicated black light to be only up to three times more attractive for *C. imicola* (Table 1).

This higher efficiency of black light is significant if taken into consideration that the most abundant *Culicoides* species in an area will not inevitably be the most competent vector species for a specific virus (Standfast et al., 1985; Paweska et al., 2002). The use of

Table 1
Culicoides species abundance and age structure (expressed as a percentage of the total catch) as determined with black and white light traps made near horses at ARC-OVI (26 October–4 November 2004)

Light source	*N*		Nulliparous female		Parous female		Freshly bloodfed female		Gravid female		Male		Total *Culicoides* (%)	
	B	W	B	W	B	W	B	W	B	W	B	W	B	W
Culicoides species														
C. imicola	16	16	51.7	53.9	42.4	40.4	0.4	0.6	2.2	1.2	3.4	4.0	48894 (95.8)	16976 (94.4)
C. bedfordi	16	16	33.0	19.1	15.0	4.4		1.5	25.8	32.8	26.3	42.2	388 (0.8)	204 (1.1)
C. leucostictus	16	16	16.1	15.4	7.8	5.6	0.2		43.7	38.5	32.2	40.6	435 (0.9)	143 (0.8)
C. pycnostictus	16	15	4.2	4.2	3.7	4.2		0.7	82.9	82.5	9.2	8.4	381 (0.8)	143 (0.8)
C. zuluensis	16	14	69.7	61.0	25.0	24.4			1.0	1.5	4.3	13.0	300 (0.6)	131 (0.7)
C. bolitinos	16	15	40.3	41.6	55.2	55.8		1.3	2.5	1.3	2.1		241 (0.5)	154 (0.9)
C. nevilli	15	16	52.8	66.7	30.8	25.0			1.1		15.4	8.3	91 (0.2)	84 (0.5)
C. magnus	14	14	53.3	52.9	43.3	44.3					3.3	2.9	90 (0.2)	70 (0.4)
C. nivosus	10	10	6.5	10.0		10.0			76.1	55.0	17.4	25.0	46 (0.1)	20 (0.1)
C. enderleini	8	3	49.1	33.3	26.4	50.0	5.7		3.8		15.1	16.7	53 (0.1)	6 (<0.1)
C. exspectator	8	9	2.6	7.1	2.6				94.7	78.6		14.3	38 (0.1)	14 (0.1)
C. similis	6	5	31.0	7.1	6.9	28.6			13.8	14.3	48.3	42.9	29 (0.1)	7 (<0.1)
C. neavei	5	4	11.1		33.3				16.7	50.0	38.9	50.0	18 (<0.1)	4 (<0.1)
C. trifascielus	3	8	33.3	33.3	33.3	58.3			33.3	8.3			6 (<0.1)	12 (<0.1)
C. nr glabripennis	4	5	37.5	33.3	62.5	33.3				33.3			8 (<0.1)	9 (0.1)
C. coarctatus	3	4	30.0	60.0	20.0	40.0			40.0		10.0		10 (<0.1)	5 (<0.1)
C. subschultzei	2	1		100							100		5 (<0.1)	1 (<0.1)
C. gulbenkiani	1	3		33.3						66.7	100		1 (<0.1)	3 (<0.1)
C. nigripennis	2		50.0								50.0		2 (<0.1)	
C. engubandei	1								100				2 (<0.1)	
C. brucei	2		100										2 (<0.1)	
C. schultzei	1								100				1 (<0.1)	
Total			50.8	52.7	41.4		0.4	0.6	3.5	2.7	4	4.9	51041	17986

N: number of collections, out of a total of 16 collections, in which this *Culicoides* species was found; B: 220 V light down draught Onderstepoort light trap equipped with 23 cm 8 W black light tube; W: 220 V light down draught Onderstepoort light trap equipped with 23 cm 8 W black light tube.

black light will greatly increase monitoring sensitivity in areas and at times marginal for *Culicoides* vectors where and when vector abundances are low, i.e. the first occurrence outside of endemic areas and sites with low vector density.

Transovarial transmission of orbiviruses is not known to occur in the genus *Culicoides* (Nunamaker et al., 1990) and therefore the number of parous individuals, and the variation in parous rate over time, is of importance in evaluating the potential vector status of a specific *Culicoides* population. Contrary to *C. sonorensis*, where it was shown that black light is biased towards the collection of parous individuals (Anderson and Linhares, 1989) no significant difference was found in the parous rates for *C. imicola* as determined by traps with black or white light sources (Table 1).

In the present study the ratio of the numbers of *Culicoides* midges collected to other insects was 1:5 for both traps.

This study confirms black light to be the preferable method for collecting and monitoring *C. imicola* populations and shows that light source may greatly influence the number of *Culicoides* that will be collected. Light source, however, is but one of a variety of factors which may influence the number of *Culicoides* specimens collected with light traps. These variables greatly impede the worldwide comparison of data and data sharing. Biases in trapping methods need to be measured and evaluated against each other so that standardized techniques for measuring the variables of vectorial capacity can be to be developed and adopted.

Acknowledgements

We thank Ms. Daphney Majatladi and Mr. Solomon Boikanyo for their technical assistance and Drs. Errol Nevill and Karin Kappmeier Green for constructive comments on earlier drafts of this manuscript. We are grateful to Edith van den Berg from the ARC-Biometry unit for assisting in the statistical analyses of the data, and the ARC-OVI for supporting this work.

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Short communication

An alternative method of blood-feeding *Culicoides imicola* and other haematophagous *Culicoides* species for vector competence studies

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Received 10 September 2004; received in revised form 14 April 2005; accepted 3 May 2005

Abstract

The use of cotton wool pads saturated with blood/virus mixture for oral infection attempts was compared to membrane feeding for the assessment of vector competence in *C. imicola* Kieffer and *C. bolitinos* Meiswinkel (Diptera, Ceratopogonidae). Although lower infection rates were obtained using pad feeding, it was possible to clearly distinguish the levels of competence between species as well as differences in virus infection rates for various serotypes of bluetongue virus. Reduced infection rates with cotton pad feeding was partly due to a smaller volume of blood meals taken up. However, the method described is likely to be useful in situations where membrane feeding is not viable to separate populations with significant differences in vector competence.

Keywords: Blood meal size; Bluetongue virus; Cotton pledget feeding; *Culicoides imicola*; Membrane feeding

1. Introduction

Since 1998 the Mediterranean Basin has been undergoing the most devastating incursion of bluetongue virus (BTV) in recorded history, with losses of more than one million sheep to date (Mellor and Wittmann, 2002; Calistri et al., 2003). During this incursion, novel Palaearctic *Culicoides* vectors, previously thought to be of limited epidemiological importance, have been implicated in the transmission of BTV in areas where the major European vector, *C. imicola* is absent (Mellor and Wittmann, 2002). In this context, BTV has been isolated in the field from pools of wild-caught specimens of two Palaearctic species complexes, the *C. obsoletus* group (Mellor and Pitzolis, 1979; Savini et al., 2003) and the *C. pulicaris* group (Caracappa et al., 2003). However, laboratory competence studies of these complexes remain limited by an extreme reluctance on the part of field collected individuals to blood-feed through membrane based

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doi:10.1016/j.vetpar.2005.05.002

systems or upon live hosts (Mellor et al., 1981; Jones et al., 1983; Mullen et al., 1985; Jennings and Mellor, 1988; Mellor, 1991). While it is known that feeding rates of up to 50% can be obtained in the *C. obsoletus* complex when they are offered cotton wool pads or pledgets saturated with a blood/virus mixture following collection (Venter and Carpenter, unpublished), this feeding method has yet to be validated as a means of assessing competence. In this study, therefore, we validate this means of feeding using the Afro-Asiatic BTV vectors *C. imicola* and the Ethiopian *C. bolitinos* as model species that are relatively easily fed through membranes, and via cotton wool pads, and are closely related to the *C. obsoletus* complex.

2. Materials and methods

2.1. Infection rates of membrane and pledget fed Culicoides

C. imicola were collected in South Africa using down-draught, 220 V light-traps equipped with 8 W UV-light tubes from January to February 2000–2004 at the ARC-OVI, Onderstepoort (25°29′S, 28°11′E; 1219 m a.s.l.). Collections were also made during January to February 2000 on Koeberg Farm near Clarens (28°32′S, 28°25′E; 1631 m a.s.l.) in South Africa where *C. bolitinos* predominates. Handling of field-collected *Culicoides* before feeding was carried-out as described previously by Paweska et al. (2003). BTV used in experiments were obtained from the OIE Reference Laboratory for bluetongue at the ARC-OVI, South Africa (serotypes 1 and 5), and from IAH Pirbright (serotype 9) (Table 1).

Field collected *Culicoides* were fed in batches of 300–500 for 30–45 min on defibrinated sheep blood spiked with one of three serotypes of BTV through a one-day-old chicken-skin membrane as described by Venter et al. (1991,1998). Stocks of BTV serotypes for oral infection studies were prepared as described by Paweska et al. (2003). In parallel, midges were also fed, immediately post-collection, on 2–3 cm^2 cotton wool pledgets saturated in blood/virus mixture (Jennings and Mellor, 1988. Post-feeding *Culicoides* were chilled and replete females separated and maintained in 250 ml un-waxed paper cups for 10 days at 23.5 °C and 50–70% relative humidity. During incubation, 10% (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 μg streptomycin and 1.25 μg/ml of fungizone) was made available via cotton wool pads. *Culicoides* surviving incubation were chilled and sorted into species before being stored individually in 1.5 ml microfuge tubes at −70 °C prior to virus isolation.

Detection of virus in *Culicoides* was carried out via application of individual homogenates to BHK-21 cell monolayers as described previously (Paweska et al., 2003). An antigen-capture ELISA (OIE, 1996) and the virus neutralization test (Venter et al., 1998) using BTV serotype-specific antisera, were used for identification of virus isolates. Virus titres in positive specimens fed on BTV 1 were calculated using the method of Kärber (1931). Fisher's exact test was used to compare the two methods of feeding and also to compare rates of virus isolation in *C. imicola* and *C. bolitinos* midges. A two-tailed Mann–Whitney test was used to compare the geometric mean virus titre in infected midges.

2.2. Comparison of blood meal sizes as determined by membrane feeding and cotton pledget methods

Feeding was carried out six times for membrane fed midges (pools ranging from 300 to 500) and five times

Table 1
Titre and passage history of BTV used

BTV serotype	Origin and strain	Passage history	Titre $\log_{10}$ $TCID_{50}$/ml
1	RSA 1958 (Biggarsberg)	50[a]E[b], 3P[c], 5BHK[d] (Howell, 1969)	6.1
5	RSA 1953 (Mossop)	50E, 2BHK, 3P, 7BHK (Howell, 1969)	6.6
9	Kosovo 2001	2E, 3BHK	7.0

[a] Number of passages.
[b] Embroynated chicken eggs.
[c] Plaque selection in green monkey kidney cells (Vero cells).
[d] Baby hamster kidney cells (BHK-21 cells).

for midges fed on cotton wool pledgets (pools ranging from 1000 to 2000). The weights of the fed midges from each method were then compared to those of control groups of unfed midges. The blood meal size was calculated from the difference in weight, taking account of the specific gravity of the whole blood (1.06).

3. Results

3.1. Infection rates of membrane and pledget fed Culicoides

Virus infected individuals were recovered from both feeding methods, irrespective of the serotypes of virus used and species of *Culicoides* examined. In all cases, virus recovery rates (i.e. infection rates) were lower in midges fed via cotton wool pads or pledgets than through membranes (Table 2).

Although virus recovery rates were lower in insects fed via cotton wool pledgets than through membranes, both methods indicated the same significant differences between *Culicoides* species and/or the BTV serotype involved. Both membrane ($P = 0.003$) and cotton pledget feeding methods ($P = 0.001$) recorded higher infection rates in *C. bolitinos* than in *C. imicola* for BTV 1. With both feeding methods (cotton pledget: $P = 0.001$; membrane feeding: $P < 0.001$) the infection rates of *C. imicola* fed with BTV 9 fed were significantly higher than for BTV 1. The infection rates of BTV 9 fed *C. imicola* were similar to BTV 5 as determined by either method (cotton pledget: $P = 0.662$; membrane feeding: $P = 0.055$). Similarly, the *C. imicola* infection rates for BTV 5 were significantly higher than with BTV 1 as determined using cotton pads ($P = 0.009$) or membrane feeding ($P = 0.001$).

Mean virus titre for midges infected with BTV 1 did not vary significantly according to the method used to feed the two species (*C. imicola*: $P = 0.787$; *C. bolitinos*: $P = 0.548$), or between the two species using the same feeding method (cotton pad: $P > 0.999$; membrane feeding: $P = 0.383$).

3.2. Comparison of blood meal sizes as determined by membrane feeding and cotton pledget methods

The blood meal size calculated from pooled *C. imicola* fed via membranes ranged from 0.023 to 0.062 μl with a mean size of 0.045 μl in six trials. Using cotton pads the blood meal sizes ranged from 0.019 to 0.038 μl with a mean of 0.03 μl. Consequently, the mean blood meal size obtained when pledget feeding was significantly smaller than when imbibing blood through a membrane ($P = 0.048$).

Table 2
Virus isolation rates and titres in *Culicoides* midges fed using two different feeding methods and three serotypes of BTV

Species	BTV serotype								
	1			5			9		
	Cotton pad	Membrane	*P*	Cotton pad	Membrane	*P*	Cotton pad	Membrane	*P*
C. imicola									
Positive BTV isolations (%)	4/587[a] (0.7)	7/402 (1.7)	0.133[NS]	8/254 (3.1)	10/115 (8.7)	0.034[S]	14/337 (4.2)	77/480 (16.0)	<0.001[S]
Range in virus tires[b]	0.7–2.4	07–2.4	0.788[NS]						
Mean titre (STD)	1.4 (0.72)	1.4 (0.73)							
C. bolitinos									
Positive BTV isolations (%)	6/104 (5.8)	3/13 (23.1)	0.061[NS]						
Range in virus titres	0.7–2.4	1.1–2.4	0.547[NS]						
Mean titre (STD)	1.4 (0.58)	1.6 (0.70)							

P = comparison between feeding methods using Fisher's exact test. [NS]: no significant difference; [S]: significant difference.

[a] Number of individuals positive/number of individuals tested.

[b] $\log_{10}$ $TCID_{50}$/midge.

4. Discussion

Blood feeding *Culicoides* using the pad or pledget feeding method produced infection rates that in comparison with membrane feeding methods and previously published data accurately reflected variations due to the serotype and titre of virus used, and also distinguished between a highly susceptible species (*C. bolitinos*) and one that was less susceptible (*C. imicola*) (Venter et al., 1998; Paweska et al., 2002). However, the overall infection rates were significantly lower in cotton pad fed midges probably due, at least in part, to a reduction in the size of the blood meal by about 30%. The data presented show that the size of the blood meal taken by *C. imicola* when using a membrane system was about 0.045 μl. This is not an absolute value as it does not take account of the elimination of excess liquid during feeding (Fujisaki et al., 1987; Leprince et al., 1989) but it can be used as a relative measure in comparisons between feeding methods, and it also equates to the blood meal volume taken from the natural host by the similarly sized BTV vector *C. brevitarsis* (Muller et al., 1982).

In a previous study using the cotton pad or pledget feeding method, Jennings and Mellor (1988) failed to record any positive results when attempting to infect the Palaearctic *C. obsoletus* complex midges with BTV (0 isolations from 441 midges tested). This difference may be related to the fact that Jennings and Mellor added sucrose to the BTV/blood mixture which increases feeding rates but also results in diversion of most of the blood meal to the blind-ending acellular crop or mid-gut diverticulum (Mellor, 1990; Venter and Carpenter, unpublished), which is not the normal route and may preclude virus establishing an infection. Alternatively, the population of *C. obsoletus* used by Jennings and Mellor (1988) had a very low level of oral susceptibility to BTV (1.6% – Mellor, 1991) and these authors may have used insufficient numbers of midges to elicit an infection rate via the pledget method. It is clearly important when using the pledget method to attempt to assess the competence of potential vectors that a sufficient number of insects is used to compensate for a two- to four-fold reduction in infection rate as shown in the present study. if that criterion is accepted, the pledget method may prove useful when attempting to assess the competence of field collected suspect vectors that are reluctant to blood feed via a membrane or on the natural host subsequent to their capture.

Acknowledgements

We thank Ina Hermanides, Isabel Wright and Hope Kekane for technical assistance and the OIE Reference Laboratory for bluetongue at the ARC-OVI for supporting this work. We also thank Mr. Ralph Burls for making the Koeberg farm available as collecting site. This study was undertaken as part of a European Union funded project (Contract number QLK2-2001-01722 – G.V.) and a DEFRA project (Contract SE 2610: S.C., H.L. & P.M.).

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Medical and Veterinary Entomology (2006) **20**, 329–334 doi: 10.1111/j.1365-2915.2006.00635.x

Oral susceptibility of South African stock-associated *Culicoides* species to bluetongue virus

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Abstract. Field-collected South African *Culicoides* species (Diptera, Ceratopogonidae) were fed on sheep blood containing bluetongue virus (BTV) represented by 13 low-passage reference serotypes: -1, -2, -4, -6, -7, -8, -9, -10, -11, -12, -13, -16 and -19. After 10 days of extrinsic incubation at 23.5°C, of the 13 serotypes used, seven were recovered from *C. (Avaritia) imicola* Kieffer and 11 from *C. (A.) bolitinos* Meiswinkel. Virus recovery rates and the mean titres for most serotypes were significantly higher in *C. bolitinos* than in *C. imicola*. In addition, BTV was recovered from three non-*Avaritia Culicoides* species, namely *C. (Remmia) enderleini* Cornet & Brunhes (BTV-9), *C. (Hoffmania) milnei* Austen (BTV-4) and *C. (H.) zuluensis* de Meillon (BTV-16). No virus could be recovered from 316 individuals representing a further 14 *Culicoides* species. In *Culicoides* species fed on blood containing similar or identical virus titres of distinct BTV serotypes, significant differences were found in virus recovery rates. The results of this study confirm the higher vector competence of *C. bolitinos* compared with *C. imicola*.

Key words. *Culicoides bolitinos*, *Culicoides imicola*, bluetongue virus, oral susceptibility.

Introduction

Bluetongue (BT) was first reported more than 128 years ago with the introduction of European breeds of sheep into southern Africa (Verwoerd & Erasmus, 2004). The causative agent, bluetongue virus (BTV), is a member of the genus *Orbivirus*, family *Reoviridae* (Borden *et al.*, 1971). Bluetongue virus causes a non-contagious, infectious, arthropod-borne disease in domestic and wild ruminants and has become established in all areas of the world where competent vector species occur (Walton, 2004). Since 1998, BTV has expanded its distribution, causing outbreaks of the disease over 800 km further north than previously recorded in southern Europe (Purse *et al.*, 2005).

In South Africa, where the majority of the sheep population consists of wool breeds, originally exotic, outbreaks of clinical disease are common and result in economic losses, either through direct mortality, or indirectly as a result of losses in condition, compromised breeding efficiency and reduced wool quality (Dungu *et al.*, 2004). Approximately one-third of the commercial sheep population of about 29 million in South Africa is vaccinated annually against bluetongue (Dungu *et al.*, 2004). Twenty-one of the 24 known BTV serotypes (Howell, 1969; Verwoerd & Erasmus, 2004) are present in South Africa (Gerdes, 2004). Although they appear to be randomly distributed in any given area during a particular season in South Africa, BTV-1, -2, -3, -4, -5, -6, -8, -11 and -24 are thought to have a high epidemic potential; BTV-9, -10, -12, -13, -16 and -19 occur regularly, and BTV-7, -15 and -18 only sporadically (Verwoerd & Erasmus, 2004).

Bluetongue virus is transmitted by certain species of *Culicoides* biting midges, the most important being *Culicoides (Avaritia) imicola* Kieffer in Asia, Africa and southern Europe (Mellor *et al.*, 1985, 2000; Boorman, 1986; Mellor, 1992; Nevill *et al.*, 1992a; Meiswinkel, 1997; Meiswinkel *et al.*, 2004a). However, evidence is growing that other *Culicoides* species may be competent field vectors of the virus in Europe (Mellor & Pitzolis, 1979; Caracappa *et al.*, 2003; Savini *et al.*, 2003). It has been demonstrated that the expansion of BTV into areas of the Mediterranean basin where *C. imicola* is rare or absent has been facilitated by at least two widespread and abundant Palearctic species groups (the *C. (Avaritia) obsoletus* and *C. (Culicoides) pulicaris* groups) (Caracappa *et al.*, 2003; De

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Liberato *et al.*, 2003; Savini *et al.*, 2003, 2005; Goffredo *et al.*, 2004; Torina *et al.*, 2004; Savini *et al.*, 2005).

In other parts of the world, a number of other species of *Culicoides* have been implicated as BTV vectors, and to date approximately 30 species of *Culicoides*, representing at least eight subgenera, are considered in the transmission of BTV (Meiswinkel *et al.*, 2004a, b).

In South Africa BTV has been isolated from field-collected *C. imicola*, *C. (Meijerehelea) pycnostictus* Ingram & Macfie, *C. expectator* (Similis group) Clastrier (Nevill *et al.*, 1992b; Meiswinkel *et al.*, 2004a), and *C. (Avaritia) bolitinos* Meiswinkel (Meiswinkel & Paweska, 2003). In addition, non-*Avaritia* *Culicoides* species from South Africa, namely *C.* (Unplaced) *bedfordi* Ingram & Macfie, *C.* (Unplaced) *huambensis* Caeiro, *C. (Culicoides) magnus* Colaço, *C. (Meijerehelea) leucostictus* Kieffer, *C. pycnostictus* and *C. milnei* were shown to be susceptible to oral infection by BTV-1 (Paweska *et al.*, 2002).

The aim of the present study was to expand on these early observations of the higher vector competence of *C. bolitinos* over *C. imicola* for BTV (Venter *et al.*, 1998; Paweska *et al.*, 2002). We report on the oral susceptibility of *C. imicola* to 13 low-passage reference serotypes of BTV in comparison with that of other livestock-associated *Culicoides* species, and especially *C. bolitinos*.

Materials and methods

Insects

Adult *Culicoides* midges for oral infection were collected using down-draught, 220 V light-traps equipped with 8 W black-light tubes (Venter & Meiswinkel, 1994) as described by Venter *et al.* (1998). Collections were carried out from February to April 2003 at the Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort 25°29′S, 28°11′E, 1219 m a.s.l., and from April to May 2003 on Koeberg Farm near Clarens (28°31′S, 28°32′E, 1631 m a.s.l) in the high-lying eastern Free State. Horses were the dominant livestock species near the four light-traps at the ARC-OVI. Ten light-traps were used to collect midges adjacent to sheep in an open-sided stable and near cattle in an open paddock on Koeberg Farm.

Viruses

Reference strains representing serotypes of BTV-1, -2, -4, -6, -7, -8, -9, -10, -11, -12, -13, -16 and -19 were obtained from the Office International des Epizooties (OIE) Reference Centre for BT, at the ARC-OVI. All were passaged 1–2 times in embryonated chicken eggs followed by 4–6 passages on baby hamster kidney-21 (BHK) cells. Stocks of these viruses for the present study were prepared as previously described (Venter *et al.*, 1998).

Feeding technique

Before feeding, the field-collected *Culicoides* were held without access to nutrients or water for 24 h at 23.5°C and a relative humidity of 50–70%. Surviving flies were subsequently fed in batches of 250–300 for 30–40 min on defibrinated sheep blood containing one of the BTV isolates using the feeding technique described by Venter *et al.* (1998). During the feeding period, lighting in the room was dimmed to ~1% daylight (~ 65 lux). A blood-virus mixture was freshly prepared immediately before feeding, as described previously (Paweska *et al.*, 2002). The blood-virus mixture was maintained at 35.5°C and stirred slowly during feeding.

Blood-engorged females were separated out on a refrigerated chill-table and incubated for 10 days at 23.5°C (Venter *et al.*, 1998). Engorged females were maintained on 5% (w/v) sucrose solution containing 500 IU penicillin, 500 μg streptomycin and 1.25 μg fungizone per mL of sucrose solution. All *Culicoides* surviving the incubation period were sorted to species on a chill-table and stored individually in 1.5-mL microfuge tubes at –70°C until assayed.

Processing of Culicoides, *virological assays and statistical analysis*

Processing of individual midges for virus microtitration assays on BHK-21 cells was carried out as described by Paweska *et al.* (2002). The identity of virus isolates was determined by a microtitre virus-neutralization procedure (House *et al.*, 1990), using type-speeific antisera produced in guinea pigs. Virus concentrations calculated according to Kärber (1931) were expressed as $\log_{10}TCID_{50}$/midge. Statistical differences between experimental groups were analysed using Fisher's exact test and/or Chi-square analysis. *P*-values of < 0.05 were used as the cut-off for statistical significance.

Results

The feeding rates for field-collected *Culicoides* females varied from 10% to 70%. A total of 9410 midges were fed in 28 attempts on 13 different serotypes of BTV, of which 4535 (48.2%), representing 19 *Culicoides* species, survived the 10-day incubation period at 23.5°C.

Of the 13 BTV serotypes used, seven (BTV-1, -10, -11, -12, -13, -16 and -19) were recovered from *C. imicola* and 11 (BTV-1, -2, -4, -7, -8, -9, -10, -11, -12, -16 and -19) from *C. bolitinos* after 10 days incubation at 23.5°C, although the numbers of the latter available for testing were lower than those of *C. imicola* (Table 1). The virus recovery rates for the same BTV serotypes were higher in *C. bolitinos* than in *C. imicola*; these differences were statistically significant ($P \leq 0.05$) for eight serotypes (BTV-1, -7, -9, -10, -11, -12, -16 and -19). Despite a relatively high virus titre of BTV-6 in the bloodmeal, this serotype could not be recovered from either *C. imicola* or *C. bolitinos* after extrinsic incubation (Table 1). The mean virus titres after 10 days incubation ranged from 1.4 to 2.4 $\log_{10}TCID_{50}$/midge in the infected *C. imicola* females and from 1.4 to 3.3 $\log_{10}TCID_{50}$/midge in *C. bolitinos* females (Table 1).

In addition, BTV was recovered from three other *Culicoides* species, namely *C. (Remmia) enderleini* Cornet & Brunhes

Table 1. Virus recovery rates and virus titres of field-collected *Culicoides* midges maintained for 10 days at 23.5°C after feeding on blood containing bluetongue virus (BTV).

BTV serotype	19	1	8	11	6	9	4	2	7	12	10	13	16
Virus titre in bloodmeal*	7.1	6.9	6.8	6.8	6.6	6.6	6.4	6.1	6.1	6.1	5.8	5.6	4.8
C. imicola													
No. positive/no. tested	1/130	19/340	0/234	13/155	0/231	0/197	0/203	0/198	0/167	1/119	1/193	1/198	2/232
(%)	(0.8)	(5.6)		(8.4)						(0.8)	(0.5)	(0.5)	(0.9)
Mean virus titre/midge†	1.4	2.4		2.1						2.3	1.9	1.7	2.4
(SD)		(0.85)		(1.55)									(0)
C. bolitinos													
No. positive/no. tested	2/12	26/98	1/38	2/4	0/13	5/61	1/49	1/63	10/24	2/4	4/11	0/10	7/26
(%)	(16.7)	(26.5)	(2.6)	(50.0)		(8.2)	(2.0)	(1.6)	(41.7)	(50.0)	(36.4)		(26.9)
Mean virus titre/midge†	3.3	2.8	1.4	3.1		2.8	1.7	2.7	2.6	2.3	2.1		3.3
(SD)	(1.24)	(1.04)		(0.06)		(1.26)			(1.44)	(0.88)	(1.54)		(0.71)
C. enderleini													
No. positive/no. tested		0/4	0/1		0/2	1/2	0/6	0/10		0/2		0/1	0/2
(%)						(50.0)							
Mean virus titre/midge†						2.4							
C. zuluensis													
No. positive/no. tested	0/1	0/10			0/1	0/1	0/4						1/2
(%)													(50.0)
Mean virus titre/midge†													2.7
C. milnei													
No. positive/no. tested							1/1						
(%)							(100)						
Mean virus titre/midge†							2.4						

*$\log_{10}TCID_{50}$/mL.
†$\log_{10}TCID_{50}$/midge.

(BTV-9), *C. milnei* (BTV-4) and *C. (Hoffmania) zuluensis* de Meillon (BTV-16); BTV virus titres in the infected midges ranged from 2.4 to 2.7 $\log_{10}TCID_{50}$/midge (Table 1). No virus could be recovered from 316 specimens, representing a further 14 *Culicoides* species, after incubation (Table 2).

The virus titres in the bloodmeal of the serotypes used in this study ranged from 4.8 $\log_{10}TCID_{50}$/mL (BTV-16) to 7.1 $\log_{10}TCID_{50}$/mL (BTV-19) (Table 1). Therefore, it was not possible to statistically compare virus recovery rates from the same *Culicoides* species for all BTV serotypes used. A comparison of virus recovery rates of BTV-1, -8 and -11 (virus titres 6.8–6.9 $\log_{10}TCID_{50}$/mL bloodmeal) in *C. imicola* shows that the recovery rates of BTV-1 and -11 were significantly higher than that of BTV-8 ($P < 0.001$), but there was no significant difference ($P = 0.243$) in the virus recovery rate of BTV-1 or -11. Similarly, in *C. bolitinos* the virus recovery rates of BTV-1 ($P = 0.001$) and BTV-11 ($P = 0.020$) were significantly higher than that of BTV-8 (Table 1). For BTV-2, -7 and -12 (virus titre of the bloodmeal = 6.1 $\log_{10}TCID_{50}$/mL), no significant differences in the virus recovery rate ($\chi^2 = 3.074$, d.f. = 2, $P = 0.215$) were found in *C. imicola*. In *C. bolitinos*, however, the virus recovery rate of BTV-7 was significantly higher ($P < 0.001$) than that of BTV-2 (Table 1). Although the virus titre of BTV-16 in the bloodmeal was relatively low (4.8 $\log_{10}TCID_{50}$/mL), the virus was recovered from seven of 26 specimens of *C. bolitinos* after extrinsic incubation (Table 1) and the virus recovery rate was significantly higher than that of viruses fed at higher titres: BTV-2 ($P = 0.001$), BTV-4 ($P = 0.002$), BTV-9 ($P = 0.037$) and BTV-8 ($P = 0.006$).

Table 2. *Culicoides* species from which no virus could be isolated after feeding on blood containing bluetongue virus (BTV) and 10 days extrinsic incubation at 23.5°C.

BTV serotype	19	1	11	6	9	4	2	12	13	16
Virus titre in bloodmeal*	7.1	6.9	6.8	6.6	6.6	6.4	6.1	6.1	5.6	4.8
Culicoides species										
C. gulbenkiani		43			26	13	57			
C. engubandei		25			10	14	22			
C. magnus		5			3	21	3			
C. leucostictus	1	4			1	13	6			
C. nivosus		1			2	3	4			6
C. subschultzei		2		2	4	3			1	
C. schultzei		2	1					1		
C. pycnostictus		1			3					
C. bedfordi		1			2				1	
C. coarctatus					2	1				
C. nevilli					2	1				
C. neavei							1			
C. huambensis						1				
C. dutoiti					1					

*$\log_{10}TCID_{50}$/mL.

Discussion

Susceptibility of a vector insect to infection by an arbovirus, and the subsequent pattern of virus replication and dissemination in its tissues, is dependent on vector genotype and pathogen (Jones & Foster, 1978; Jennings & Mellor, 1987; Tabachnick, 1991; Mecham & Nunamaker, 1994; Fu *et al.*, 1999; Wittmann, 2000; Mellor, 2004; Mullens *et al.*, 2004) and on environmental factors (Mullens *et al.*, 1995; Tabachnick, 1996; Mellor *et al.*, 1998; Wittmann, 2000; Wittmann *et al.*, 2002; Campbell *et al.*, 2004). Laboratory demonstration of the potential of a particular species to support pathogen replication and transmission is one of the critical components aiding determination of vectorial capacity, which also depends upon biting rate, host selection, vector survivorship and the extrinsic incubation period of the virus (Mullens, 1992; Mullens *et al.*, 2004).

As a result of differences in the virus titres of BTV serotypes used to spike bloodmeals in this study, it was not possible to compare the virus recovery rates for the same *Culicoides* species for all the different viruses. However, where infectivity of BTV serotypes was the same or comparable in bloodmeals, significant differences in virus recovery rates were found (Table 1). Field populations of *Culicoides* species may vary greatly in respect to susceptibility to BTV infection and it was demonstrated that some field populations of *Culicoides* species might be completely refractory to infection by some serotypes (Ward, 1994; Mellor, 2004). The relatively low virus recovery rates found in this study in *C. imicola* for most of the BTV serotypes do not necessarily reflect a low vectorial capacity. For example, *C. brevitarsis* is considered the most competent vector of BTV in Australia, although the highest prevalence of infection determined in this species by feeding on viraemic sheep was only 1.5% (Bellis *et al.*, 1994). In the field, the low infection rate with BTV in *C. brevitarsis* is likely to be compensated for by very high biting rates, leading to the successful transmission of the virus (Muller, 1985; Mullens *et al.*, 2004). As in Australia, the high abundance of *C. imicola* in South Africa (Venter *et al.*, 1996; Meiswinkel *et al.*, 2004a) on livestock can compensate for the low vector competence demonstrated in this study.

A potential problem with using field-collected *Culicoides* in assessing susceptibility for a virus under laboratory conditions is that some specimens may have been infected with unknown field viruses. However, virus recovery from field-collected insect vector populations is generally very low (Walter *et al.*, 1980; Gerry *et al.*, 2001). In a countrywide survey in South Africa conducted from 1979 to 1985, BTV was isolated on 526 occasions from a total of 4506 *Culicoides* pools, each consisting of 100–100 000 individuals (Nevill *et al.*, 1992b). As a conservative estimate, even if all the pools consisted of only 1000 individuals, this would result in a field infection prevalence of less than six of 10 000 midges. Therefore, the virus recovery rates recorded for *Culicoides* in the present study were most likely the result of laboratory infection. This is supported by the fact that the identities of all BTV serotypes recovered from midges in this study were the same as those used in the experimental infections.

We demonstrated that, compared with the Onderstepoort population of *C. imicola*, the Clarens population of *C. bolitinos* was significantly more susceptible to infection with different BTV serotypes and supported their replication to higher titres. *C. imicola* and *C. bolitinos* ingest similarly sized bloodmeals and it has been demonstrated that they take up similar amounts of an orbivirus during an artificial feeding (Paweska *et al.*, 2002; Venter *et al.*, 2005). Results of previous vector competence studies in South Africa have shown that, compared with *C. imicola*, the Clarens population of *C. bolitinos* not only supported replication of BTV-1, -3 and -4 to higher titres (Venter *et al.*, 1998), but also had a significantly higher transmission potential for BTV-1 over a range of different incubation periods and temperatures (Paweska *et al.*, 2002).

In the present study BTV was recovered from *C. milnei*, *C. zuluensis* and *C. enderleini*. All three of these *Culicoides* species are relatively abundant in South Africa and are known to attack livestock (Nevill *et al.*, 1992a; Meiswinkel *et al.*, 2004a). While the latter two species are widespread in South Africa, *C. milnei* is considered to be localized in its distribution (Nevill *et al.*, 1992a). Bluetongue virus has been isolated from field-collected specimens of *C. milnei* in Kenya (Walker & Davies, 1971). Based on overall abundance in light-traps, geographical distribution, virus isolation from field-collected *Culicoides*, host preference and larval habitats, Nevill *et al.* (1992a) rated both *C. zuluensis* and *C. enderleini* as having high vector potentials, whereas *C. milnei* was considered to have a low vector potential. The possible involvement of non-*Avaritia* species in the transmission of BTV in South Africa has been reported in greater detail earlier (Paweska *et al.*, 2002) and reflects findings made elsewhere in the world (Meiswinkel *et al.*, 2004b).

The virus titres in some individual midges determined in this study after extrinsic incubation were ≥ 2.5 $\log_{10}$TCID$_{50}$/midge (Table 1). Titres of this magnitude of an orbivirus have been postulated as an indicator of a fully disseminated 'transmissible' infection (Jennings & Mellor, 1987). As this infectious threshold helps to distinguish between transmissive and non-transmissive individuals, results of laboratory infection studies can provide data of epidemiological importance. However, this threshold was derived from the *C. sonorensis*/BTV laboratory model and it is unlikely that it can be directly applied to other *Culicoides* species, especially those that are smaller in size than *C. sonorensis*. By contrast with the highly standardized *C. sonorensis*/BTV model, vector competence studies on field-collected *Culicoides* cannot be fully controlled; *Culicoides* are a biologically highly diverse genus and thus extrapolation of vector competence data from one species to others is not recommended (Tabachnick, 1992). The higher mean virus titres found in *C. bolitinos* after incubation may, however, be indicative of a higher transmission potential in this species.

The correlation between the level of viraemia and infection prevalence, as well as the minimum level of viraemia necessary to infect South African *Culicoides* vectors with BTV, is unknown. Based on the bloodmeal size of *C. imicola* and *C. bolitinos* (Venter *et al.*, 2005), it can be calculated that 5 $\log_{10}$ID/mL of blood is theoretically needed to expose vectors to approximately 1 TCID$_{50}$. It is therefore possible that the virus recovery rates as determined in this study, especially for the lower infectivity titres of BTV used, will be much lower than the susceptibility rate of the *Culicoides* species analysed.

Our present findings confirm that the Clarens population of *C. bolitinos* is significantly more susceptible to oral infection with different serotypes of BTV than the Onderstepoort population of *C. imicola*. Should our results reflect the same level of genetic susceptibility to infection with this virus in other populations of *C. imicola* and *C. bolitinos*, the role of the latter in BTV transmission in South Africa might be considerable, despite its generally lower abundance compared with *C. imicola*. Nevertheless, present results contribute to a better understanding of BT epidemiology in cooler areas of South Africa, where the known vector of BTV, *C. imicola*, is virtually absent.

Acknowledgements

We thank Ina Hermanides, Isabel Wright, Sandra Prinsloo and Hope Kekana for technical assistance and the ARC-OVI for supporting this work. We also thank Ralph Burls for making Koeberg Farm available as a collecting site. This work was supported by a grant from the European Union (contract no. QLK2-2001–01722).

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Accepted 19 May 2006
First published online 2 August 2006

Medical and Veterinary Entomology (2007) **21**, 239–247

Replication of live-attenuated vaccine strains of bluetongue virus in orally infected South African *Culicoides* species

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Abstract. Field-collected South African *Culicoides* (Diptera, Ceratopogonidae) were fed on sheep blood containing 16 live-attenuated vaccine strains of bluetongue virus (BTV) comprising serotypes -1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -16 and -19. After 10 days extrinsic incubation at 23.5 °C, 11 and seven of the 16 BTV serotypes used were recovered from *Culicoides (Avaritia) imicola* Kieffer and *Culicoides (A.) bolitinos* Meiswinkel, respectively. One serotype was also recovered from *Culicoides (Remmia) enderleini* Cornet & Brunhes. Bluetongue virus recovery rates and the mean titres for most serotypes were significantly higher in *C. bolitinos* than in *C. imicola*. Significant differences were found in virus recovery rates from *Culicoides* species fed on blood containing similar or identical virus titres of different BTV serotypes. In addition, we demonstrated that a single passage of live-attenuated BTV-1, -2, -4, -9 and -16 through the insect vector, followed by passaging in insect cells, did not alter its infectivity for *C. imicola* and that the oral susceptibility of *C. imicola* to the attenuated vaccine strains of BTV-1, -4, -9 and -16 remained similar for at least three consecutive seasons.

Key words. *Culicoides bolitinos*, *Culicoides imicola*, bluetongue virus, live-attenuated vaccine strains, oral susceptibility, South Africa.

Introduction

Bluetongue virus (BTV), genus *Orbivirus*, family *Reoviridae* (Borden *et al.*, 1971), causes a non-contagious, infectious, arthropod-borne disease in domestic and wild ruminants. Bluetongue virus has become established in all areas of the world in which competent vector species occur (Walton, 2004) and most recently has expanded its distribution in Europe, where it has caused serious outbreaks of clinical bluetongue (BT) disease (Purse *et al.*, 2005). Since September 2006, outbreaks of BTV have been confirmed as far north as the Netherlands, Belgium, Germany and northern France (OIE, 2006).

Bluetongue was first reported more than 130 years ago with the introduction of European breeds of sheep into southern Africa (Verwoerd & Erasmus, 2004). In South Africa, where the majority of sheep populations consist of originally exotic wool breeds, outbreaks of clinical disease are common and result in economic losses, either through direct mortality, or indirectly as a result of a loss in condition, compromised breeding efficiency and reduction in wool quality (Dungu *et al.*, 2004a). Twenty-one of the 24 known BTV serotypes (Howell, 1969) are present in South Africa and currently only types 20 and 21 are considered to be exotic (Gerdes, 2004). Approximately one-third of a commercial sheep population of about 29 million in South Africa is vaccinated annually against BT (Dungu *et al.*, 2004a).

Bluetongue virus is transmitted by certain species of *Culicoides* biting midges, the most important being *Culicoides (Avaritia) imicola* Kieffer in Asia, Africa and southern Europe (Mellor *et al.*, 1985, 2000; Meiswinkel *et al.*, 2004a, b). However, evidence, is growing that other *Culicoides* spp., belonging to the *obsoletus* and *pulicaris* complexes, may be competent field vectors of the virus in Europe (Mellor & Pitzolis,

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1979; Savini *et al.*, 2005) and across the world. Approximately 30 *Culicoides* spp., representing at least eight subgenera, are considered to be involved in the transmission of BTV (Meiswinkel *et al.*, 2004a, b). In South Africa, at least 10 species, representing eight subgenera, of the more than 120 species of *Culicoides* identified at present have been implicated in the transmission of BTV and related orbiviruses (Nevill *et al.*, 1992; Venter *et al.*, 1998; Paweska *et al.*, 2002, 2005; Meiswinkel & Paweska, 2003; Meiswinkel *et al.*, 2004b; Paweska & Venter, 2004).

Because of BTV's ubiquitous and broad host range, the multiplicity of serotypes that may be circulating at any one time, as well as the wide distribution and seasonal occurrence of vector species of *Culicoides*, the eradication of BTV in endemic regions remains a challenge. Attenuated live-virus vaccines have been used to control BT in endemic areas of South Africa for many years. Concerns about the induction of clinical signs in some sheep breeds, the development of viraemia with live virus vaccines and the high potential for transmission by *Culicoides* in the field were most recently addressed by Veronesi *et al.* (2005). There is also a fear that vaccine viruses may revert to more pathogenic strains through passage in vectors and may then be transmitted in the field (Walton, 1992). *Culicoides (Monoculicoides) sonorensis* Wirth & Jones, infected through feeding on vaccinated sheep, has been shown to transmit attenuated vaccine virus to susceptible animals (Foster *et al.*, 1968). Another concern is that reassortment between vaccine and wild-type viruses may occur in the field and give rise to strains with novel virulence characteristics (Samal *et al.*, 1987; El Hussein *et al.*, 1989; Kirkland & Hawkes, 2004). Therefore, data on oral susceptibility and vector competence of *Culicoides* midges for live-attenuated vaccine strains of orbiviruses are important for risk assessment analysis, particularly when intervention with live-attenuated vaccines is to be considered in regions vulnerable to virus incursions. Paweska *et al.* (2003) have shown replication of the tissue culture-attenuated vaccine strains of African horse sickness virus in various species of stock-associated South African *Culicoides* spp., including *C. imicola* and *C. bolitinos*. Active circulation of BT vaccine virus serotype-2 among unvaccinated cattle in central Italy has also been documented by Ferrari *et al.* (2005). Adaptation of BTV to mammalian cell culture, as in the development of live-virus vaccines, can result in altered behaviour of the virus (Gard, 1987) and infectivity for vectors and changes in tissue tropism in the vertebrate host are two possible altered characteristics (Bellis *et al.*, 1994). A striking example of this phenomenon is the demonstration that only tissue-culture attenuated strains of BTV were shown to cross the ruminant placenta and cause teratological effects of the foetal central nervous system in sheep and cattle (Griner *et al.*, 1964; Dungu *et al.*, 2004b; Verwoerd & Erasmus, 2004).

Although annual and massive immunization of sheep with live polyvalent BT vaccines has been practised in South Africa for decades, data on the vector competence of *Culicaides* midges for strains that are included in the current commercial vaccines are very limited. The practical difficulties with the implementation of experimental procedures to determine if attenuated orbiviruses can be transmitted by insects from vaccinated to non-vaccinated vertebrate hosts and, with possible reversion to virulence during a number of mammal-insect replication cycles, have been discussed by Paweska *et al.* (2003). However, the results of preliminary studies by Venter *et al.* (2004) indicated that the South African *Culicoides* species, *C. imicola* and *Culicoides bolitinos*, are susceptible to oral infection with live-attenuated strains of BTV-1, -4, -9 and -16.

The aim of this study was to expand on these initial observations and to determine the oral susceptibility of *C. imicola* and other livestock-associated *Culicoides* spp. to all cell culture-attenuated BTV vaccine strains currently used for the vaccination of sheep in southern Africa.

In addition, we aimed to assess the effect of a single passage of BTV in *C. imicola*, followed by passaging in insect cells, on oral infectivity in *C. imicola* and to determine interseasonal variations in oral susceptibility of *Culicoides* spp. to infection with BTV.

Materials and methods

Insects

Adult *Culicoides* spp. were collected using down-draught, 220-V light traps equipped with 8-W black-light tubes as described by Venter *et al.* (1998). Collections were carried out annually during January–April from 2002 to 2004 at the ARC-Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort (25°29′S, 28°11′E; 1219 m above sea level [a.s.l.]) and on Koeberg Farm near Clarens (28°32′S, 28°25′E; 1631 m a.s.l.) in the eastern Free State. Horses were the most abundant livestock found near the four light traps at the ARC-OVI. Ten light traps were used to collect midges adjacent to sheep in an open-sided stable and near cattle in an open paddock on Koeberg Farm.

Virus

The strain identification, origin and passage history of the attenuated vaccine strains of BTV used are listed in Table 1. Stocks of vaccine and reference viruses for oral infection studies were grown in baby hamster kidney -21 cells (BHK-21 cells), titrated and stored in 10% foetal bovine serum in 1-mL aliquots at −70 °C. Aliquots of virus stocks for oral infection were titrated during the experiments as described by Venter *et al.* (1998). The virus titres of the bloodmeals used for oral infection are shown in Table 2.

To determine if passage of the live-attenuated vaccine viruses through an insect vector would alter virus infectivity to infect vectors, field-collected midges were fed on blood spiked with midge-isolates of the vaccine strains. Because of the relatively low BTV recovery rates from orally infected *C. imicola*, these midge-isolates were obtained from field-collected *C. imicola* females that had been intrathoracically inoculated with one or other of the vaccine strains of BTV-1, -2, -4, -9 and -16. Of 2892 field-collected *C. imicola* inoculated with the vaccine strains of BTV, a total of 911 (31.5%) survived the 1-day incubation period at 23.5 °C. After incubation, surviving *C. imicola* females were pooled (*c.* 180 individuals/BTV serotype) and these pools

Table 1. Identification, origin and passage history of bluetongue virus live-attenuated vaccine strains (Erasmus, B.J. 2006, Deltamune, personal communication).

Bluetongue virus serotype	Strain identification	Origin	Passage history
1	Biggarsberg/8012	SA 1958	50E 3P 4BHK
2	Vryheid/5036	SA 1958	50E 3P 4BHK
3	Cyprus/8231	Cyprus 1944	45E 2BHK 3P 5BHK
4	Theiler/79043	SA, ~1900	60E 3P 9BHK
5	Mossop/4868	SA 1953	50E 2BHK 3P 6BHK
6	Strathene/5011	SA 1958	60E 3P 7BHK
7	Utrecht/1504	SA 1955	60E 3P 7BHK
8	Camp/8438	SA 1937	70E 3BHK 10P 7BHK
9	University Farm/2766	SA 1942	50E 2BHK 3BHK 7P
10	Portugal/2627	Portugal 1956	81E 6BHK
11	Nelspoort/4575	SA 1944	35E 3P 5BHK
12	Estansia/75005	SA 1941	55E 3P 4BHK
13	Westlands/7238	SA 1959	45E 2BHK 3P 4BHK
14	Kolwani/89/59	SA 1959	60E 3P 5BHK
16	Pakistan/7766	Pakistan	37E 3P 2BHK 1Vero
19	143/76	RSA 1976	29E 3P 3BHK

SA, South Africa; E, number of passages in eggs; P, number of plaque selections; BHK, number of passages in baby hamster kidney cells; Vero, number of passages in green monkey kidney cells.

titrated in BHK-21 cells to confirm infection and titre of virus present/pool. As the titre of virus obtained in this way was invariably too low to use in vector oral infection studies, the virus in such midge isolates was amplified by passaging in *Aedes albopictus* C6/36 cells until the titre reached at least 7.0 $\log_{10}$TCID$_{50}$/mL. To achieve virus titres between 5.8 and 6.8 $\log_{10}$ TCID$_{50}$/mL in the bloodmeal the various midge isolates were passaged two to four times on *Ae. Albopictus* C6/36 cell cultures. The oral susceptibility of field-collected *C. imicola* for these vector and *Ae. albopictus* C6/36 cell cultures passaged viruses was compared with that of similar titres of the same vaccine strains of the virus, prior to passage through the vector.

Feeding technique

Before feeding, the field-collected *Culicoides* spp. were held without access to nutrients or water for 24 h at 23.5 °C and a relative humidity (RH) of 50–70%. Surviving flies were subsequently fed in batches of 250–300 for 30–45 min on defibrinated sheep blood, containing one of the BTV isolates, through a 1-day-old chicken skin membrane (Venter *et al.*, 1998). During feeding, lighting in the room was dimmed to ~1% daylight (~65 lux). Each blood-virus mixture was prepared immediately before feeding (Paweska *et al.*, 2002). The mixture was maintained at 35.5 °C and stirred constantly during feeding.

Blood-engorged females were selected on a refrigerated chill-table and incubated at 23.5 °C for 10 days. Engorged females were maintained on 5% (w/v) sucrose solution containing 500 IU penicillin, 500 μg streptomycin and 1.25 μg fungizone per mL. All *Culicoides* spp. surviving the incubation period were sorted to species on a chill-table and stored individually in 1.5 mL microfuge tubes at −70 °C until assayed.

Processing of Culicoides, *virological assays and statistical analysis*

Flies were assayed for virus immediately after feeding on blood-virus mixtures or after 10 days of extrinsic incubation at 23.5 °C. Processing of samples and virus microtitration assays in BHK-21 cells were performed as described by Paweska *et al.* (2002). The identity of all virus isolates was determined by a microtitre virus-neutralization procedure (House *et al.*, 1990) using type-specific antisera produced in guinea pigs. Virus concentrations, calculated by the method of Kärber (1931), were expressed as $\log_{10}$TCID$_{50}$/midge. Statistical differences between experimental groups for categorical data were analysed using Fisher's exact test and/or chi-square analysis. *P*-values of < 0.05 were used as the cut-off for statistical significance.

Results

Attenuated vaccine strains

During the summer of 2002–2003 the live-attenuated vaccine strains of BTV-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -16 and -19 were used in oral susceptibility studies. A total of 5359 (54.5%) *Culicoides* survived the 10-day incubation period at 23.5 °C. The number of midges assayed successfully after incubation are shown in Table 2.

Virus recovery results in midges that survived the 10-day incubation indicated that at least 11 of the 16 live-attenuated BTV strains used (i.e. BTV-1, -3, -4, -5, -6, -7, -8, -10, -12, -13 and -19) had replicated in *C. imicola*. Significantly lower numbers of *C. bolitinos* were tested and virus recovery results from incubated midges indicated that seven of the 16 live-attenuated BTV

Table 2. Virus recovery rates and titres in field-collected *Culicoides imicola*, *C. bolitinos* and *C. enderleini* maintained for 10 days at 23.5 °C after feeding on blood containing vaccine strains of bluetongue virus.

	Bluetongue virus serotype															
	5	4	1	3	6	16	2	10	13	19	7	9	12	14	11	8
Virus titre in bloodmeal*	6.6	6.3	6.1	6.1	6.1	6.1	5.8	5.8	5.8	5.8	5.6	5.3	5.3	5.1	4.8	4.6
C. imicola	50/473	3/342	7/402	1/165	1/226	0/218	0/291	6/200	1/125	1/122	4/164	0/343	4/234	0/190	0/146	9/142
n positive/*n* tested (%)	(10.6)	(0.9)	(1.7)	(0.6)	(0.4)			(3.0)	(0.8)	(0.8)	(2.4)		(1.7)			(6.3)
Mean virus titre/midge†	3.9	2.0	1.3	4.2	2.4			1.3	1.9	1.9	3.0		2.2			3.2
(SD)	(1.20)	(0.77)	(0.73)									(0.60)		(1.82)		(1.71)
Range in virus titre	1.1–6.2	1.1–2.4	0.7–2.4					0.7–1.9			2.4–3.7		0.7–4.4			0.7–4.9
C bolitinos	10/16	1/33	3/13	1/3	1/11	0/34	0/7	1/22	0/12	0/13	0/18	0/55	5/43	0/6	0/29	0/18
n positive/*n* tested (%)	(62.5)	(3.0)	(23.1)	(33.3)	(9.1)			(4.5)					(11.6)			
Mean virus titre/midge (SD)	4.4 (1.26)	3.4	1.6 (0.70)	1.7	2.4			2.2					3.7 (1.02)			
Range in virus titre	2.4–6.2		1.1–2.4										2.4–4.7			
C. enderleini	0/1		0/1	1/5				0/3						0/1		0/1
n positive/*n* tested (%)				(20.0)												
Mean virus titre/midge				4.7												

*$Log_{10}TCID_{50}$/mL.
†$Log_{10}TCID_{50}$/midge.
SD, standard deviation.

vaccine strains used (i.e. BTV-1, -3, -4, -5, -6, -10 and -12) had replicated or persisted in *C. bolitinos* (Table 2). Whereas BTV-2, -9, -11, -14 and -16 were not recovered from either *C. imicola* or *C. bolitinos*, BTV-7, -8, -13 and -19 were only recovered from *C. imicola* in this trial (Table 2). Virus recovery rates, ranging from 62.5% (BTV-5) to 3.0% (BTV-4) in *C. bolitinos*, were higher than those in *C. imicola*. For BTV-1 ($P = 0.003$), BTV-3 ($P = 0.036$), BTV-5 ($P < 0.001$) and BTV-12 ($P = 0.006$) virus recovery rates from *C. bolitinos* were significantly higher than those from *C. imicola*.

The virus titres in the bloodmeal of the serotypes used in this study ranged from 4.6 (BTV-8) to 6.6 $\log_{10}TCID_{50}$/mL (BTV-5) (Table 2) and it is therefore not possible to compare virus recovery rates from the same *Culicoides* species for all the BTV serotypes. A comparison of virus recovery rates of BTV-1, -3, -6 and -16 (virus titre 6.1 $\log_{10}TCID_{50}$/mL of bloodmeal) shows that the recovery rates of these serotypes were not significantly different in *C. imicola* (Table 2). In *C. bolitinos* the virus recovery rate of BTV-1 was significantly higher than that of BTV-16 ($P = 0.018$) (Table 2). A comparison of virus recovery rates of BTV-2, -10, -13 and -19 (virus titre of the bloodmeal = 5.8 $\log_{10}TCID_{50}$/mL) in *C. imicola* showed that the recovery rate of BTV-10 was significantly higher than that of BTV-2 ($P = 0.004$). Similar comparisons of the recovery rates of BTV-9 and -12 (virus titre of the bloodmeal = 5.3 $\log_{10}TCID_{50}$/mL) showed that the recovery rate of BTV-12 was significantly higher in both *C. imicola* ($P = 0.027$) and *C. bolitinos* ($P = 0.014$). Although the virus titre of BTV-8 in the bloodmeal was relatively low (4.6 $\log_{10}TCID_{50}$/mL), the virus was recovered from nine of 142 specimens of *C. imicola* after extrinsic incubation (Table 2) and the virus recovery rate was significantly higher than that for those viruses fed at higher titres: BTV-1 ($P = 0.009$); BTV-2 ($P < 0.001$); BTV-3 ($P = 0.007$); BTV-4 ($P = 0.001$); BTV-6 ($P = 0.001$); BTV-9 ($P < 0.001$); BTV-11 ($P = 0.002$); BTV-12 ($P = 0.037$); BTV-13 ($P = 0.022$); BTV-14 ($P < 0.001$); BTV-19 ($P = 0.023$), and BTV-16 ($P < 0.001$).

One of five specimens of *Culicoides enderleini* which had fed on BTV-3 was positive for BTV after incubation. The virus titre in this midge was 4.7 $\log_{10}TCID_{50}$/midge after the intrinsic incubation period (Table 2). No virus could be recovered from an additional 14 *Culicoides* spp. assayed after incubation; however, the number of midges successfully examined was extremely small and these observations should not be considered definitive (Table 3).

Vector and Ae. albopictus *C6/36 cell cultures passaged vaccine virus strains*

During the summer of 2003–2004, batches of field-collected *C. imicola* were fed on either the vaccine strains of BTV-1, -2, -4, -9 and -16 or on vector-passaged examples of the same strains. A total of 3162 individuals (45.2%) survived the 10-day incubation period at 23.5 °C. The number of midges assayed after incubation, the number of passages on *Ae. albopictus* C6/36 cell cultures, the titre of the bloodmeals and the virus recovery rate from midges fed with the various virus isolates are

Table 3. Oral susceptibility of field-collected *Culicoides imicola* to bluetongue vaccine virus strains and to vector/*Aedes albopictus* C6/36 cell culture passaged bluetongue vaccine strains. Midges tested after 10 days of incubation at 23.5 °C.

	BTV1		BTV2		BTV4		BTV9		BTV16	
Virus	A*	MI(2)†	A	MI(4)	A	MI(4)	A	MI(3)	A	MI(4)
Virus titre of bloodmeal‡	6.1	6.3	5.8	6.1	6.3	6.6	5.3	6.8	6.1	5.8
n positive/*n*	7/244	0/268	1/168	1/187	0/304	1/265	2/170	2/455	1/374	1/157
Percentage testing positive	2.9		0.6	0.5		0.4	1.2	0.4	0.3	0.6
Mean virus titre/midge§	1.2		0.7	1.4		1.4	1.2	1.1	0.7	1.4
Range	0.7–1.7						0.7–1.7	0.7–1.4	0.7	

*Attenuated virus strain.
†Midge isolate and number of passages on C6/36 cells.
‡$Log_{10}TCID_{50}$/mL.
§$Log_{10}TCID_{50}$/midge.
BTV, bluetongue virus.

shown in Table 3. The virus recovery rate was significantly higher in *C. imicola* fed on the vaccine strain of BTV-1 ($P = 0.005$) than on an isolate of the same virus passaged twice through *C. imicola* and twice through *Ae. albopictus* C6/36 cell cultures. There was no significant difference in virus recovery rates from *C. imicola* fed on vaccine strains of BTV-2 ($P = 1.000$), BTV-4 ($P = 0.466$), BTV-9 ($P = 0.299$) and BTV-16 ($P = 0.504$) compared with those from *C. imicola* fed onh the same viruses that had been subjected to vector/insect cell passages prior to feeding.

Interseasonal stability in oral susceptibility to BTV infection in Culicoides

Vaccine strains of BTV-1, -4, -9 and -16 were used in this study because of their involvement in the outbreaks of BT in Europe prior to 2001 and consequent intense international interest (Venter *et al.*, 2004). These BTV serotypes were used, in the current work, for the oral infection of field-collected *C. imicola* for three consecutive summers. Virus recovery results from midges fed in the 2002–2003 and 2003–2004 seasons were compared with one another and with the published results from the 2001–2002 season (Venter *et al.*, 2004), but no significant differences in virus recovery rates between the three seasons were found ($P > 0.07$ in all cases) (Table 5).

Virus recovery from *C. imicola* fed on the vaccine strain of BTV-1 was significantly higher than that from midges fed on the vaccine strains of BTV-4 ($\chi^2 = 12.970$, d.f. $= 1$, $P < 0.001$), BTV-9 ($\chi^2 = 16.929$, d.f. $= 1$, $P < 0.001$) and BTV-16 ($\chi^2 = 23.509$, d.f. $= 1$, $P < 0.001$). Although the virus titre in the bloodmeal for the vaccine strain of BTV-9 was one log lower than for the other strains, there were no significant differences in virus recovery rates from *C. imicola* fed on BTV-4, -9 and -16 ($\chi^2 = 5.870$, d.f. $= 2$, $P = 0.053$) (Table 5).

Discussion

The present study showed that *C. imicola* and *C. bolitinos* can become infected and support the replication of at least 12 of the 16 attenuated BTV strains used in the current production of commercial polyvalent vaccines in South Africa. The relatively low virus recovery rates found in *C. imicola* for most of the highly attenuated serotypes used is comparable with that showed for various other low passaged BTV serotypes (Venter *et al.*, 1998, 2006; Paweska *et al.*, 2002). Low recovery rates of BTV from *C. imicola* do not necessarily reflect low vectorial capacity. In Australia, low BTV recovery rates from *Culicoides brevitarsis* are compensated for by very high biting rates, resulting in this species being the most important vector in that country (Muller *et al.*, 1982; Mullens *et al.*, 2004). Similarly, the very high abundance of *C. imicola* near livestock in South Africa can compensate for its low vector competence (Meiswinkel *et al.*, 2004b).

As in previous studies (Venter *et al.*, 1998, 2006; Paweska *et al.*, 2002) we have demonstrated here that, compared with the Onderstepoort population of *C. imicola*, the Clarens population of *C. bolitinos* is significantly more susceptible to oral infection with BTV and supported replication of several serotypes to higher titres. Should these results reflect similar levels of genetic susceptibility to infection with BTV in other populations of *C. imicola* and *C. bolitinos*, the role of the latter species in BTV transmission in South Africa will probably become much more important than was previously thought, despite the fact that it is 10 times less abundant than *C. imicola* (Venter *et al.*, 1996).

In the current study, in addition to *C. imicola* and *C. bolitinos*, the vaccine strain of BTV-3 was also recovered after the extrinsic incubation period from a non-*Avaritia* livestock-associated species, namely *C. enderleini*. Based on abundance in light traps, geographical distribution, host preference and larval habitat, Nevill *et al.* (1992) rated *C. enderleini* as having a high potential vector capacity and our work tends to support that assertion. The possible involvement of non-*Avaritia Culicoides* species in the transmission of BTV in South Africa has been reported in detail (Paweska *et al.*, 2002) and reflects similar findings made elsewhere in the world where such species may be of major importance in the transmission of BTV (Meiswinkel *et al.*, 2004a).

As a result of differences in the virus titres of the BTV serotypes used to spike bloodmeals in this study, it was not possible to compare the virus recovery rates for the same *Culicoides* species for all the different viruses. However, where infectivity of BTV serotypes was the same or similar, such comparisons in

Table 4. *Culicoides* species and number of individuals tested from which virus could not be isolated after feeding on blood containing vaccine strains of bluetongue virus after 10 days of extrinsic incubation at 23.5 °C.

	Bluetongue virus serotype														
	5	4	1	3	6	16	2	10	13	19	7	9	12	14	11	8
Virus titre in bloodmeal*	6.6	6.3	6.1	6.1	6.1	6.1	5.8	5.8	5.8	5.8	5.6	5.3	5.3	5.1	4.8	4.6
C. nivosus			10	2	1	5	4	2	2		2			1		1
C. pycnostictus		7	2	3		6	2	1		1		3				2
C. leucostictus		7	3		1	2	3	1				5	1	1		1
C. zuluensis		2	5	7	1	2		2		1	1	3				
C. subschultzei		2	1				2						2			
C. neavei		1	1				1			1						
C. coarctatus			2			1										
C. gulbenkiani			1			1										1
C. engubandei			1											1		
C. bedfordi		1										1				
C. schultzei			1													
C. nigripennis															1	
C. magnus						1										
C. nevilli	1															

*$Log_{10}TCID_{50}$/mL.

virus recovery rates were made and significant differences were sometimes found (Table 2). These findings are in agreement with those of Mecham & Nunamaker (1994), who reported significant differences in infection rates in *C. sonorensis* colonies when using five different BTV serotypes. Although Mecham & Nunamaker (1994) did not demonstrate significant differences in overall infection rates between two colonies of *C. sonorensis*, they did show significant differences in the infection rates of individual serotypes between the colonies. The significance of this is not clear but field populations of *Culicoides* spp. may vary greatly in genetic susceptibility to BTV infection. Indeed, it has been demonstrated that some populations of a species might be completely refractory to infection by some serotypes of the virus (Ward, 1994; Mellor, 2004; Carpenter *et al.*, 2006). In the present study, which used populations of *C. imicola* that have been shown to express low levels of competence for BTV, it may be that the differences recorded are the result of the probability of a competent individual being included in the small sample sizes and surviving the incubation period. If so, this demonstrates the importance of using large sample sizes and the necessity of statistical analyses for studies of this type (Mecham & Nunamaker, 1994).

The attenuated viruses used in this work were originally selected as vaccine strains by the vaccine manufacturer because replication in the vertebrate host yielded a viraemia of not more than 10^3PFU/mL of blood in sheep, which was considered to be too low to infect vector *Culicoides* (Dungu *et al.*, 2004a). The minimum level of viraemia necessary to infect South African *Culicoides* veetors with BTV is; however, not known. Based on the bloodmeal size of *C. imicola* and *C. bolitinos*, which ranges from 0.01 μL to 0.060 μL, it can be calculated that a viraemia of $10^5 log_{10}$ID virus per mL is required to expose each vector to approximately one $TCID_{50}$ of virus (Venter *et al.*, 2005). In this context, Muller *et al.* (1982), working in Australia, showed the volume of the bloodmeal taken by a single *C. brevitarsis* to be about 0.03 μL, and yet these authors were able to obtain an infection rate of 0.2% in midges fed on cattle with a BTV-20 viraemia of approximately 10^2 $TCID_{50}$/mL. This suggests that each midge that received one $TCID_{50}$ of virus became infected. Even more extraordinary, in the U.S.A., Bonneau *et al.* (2002) succeeded in infecting some *C. sonorensis* by feeding them on an aviraemic BTV-infected sheep. As the lower detection limit

Table 5. Comparison of virus recovery rates, after 10 days of incubation at 23.5 °C, in *Culicoides imicola* fed on the live-attenuated vaccine strains of bluetongue virus (BTV), over three consecutive summer seasons.

Season	2002*	2003	2004
BTV 1 (6.1 $log_{10}TCID_{50}$/mL) *n* positive/*n* tested (%)	22/712 (3.1)	7/402 (1.7)	7/244 (2.9)
BTV 4 (6.3 $log_{10}TCID_{50}$/mL) *n* positive/*n* tested (%)	10/886 (1.1)	3/342 (0.9)	0/304
BTV 9 (5.3 $log_{10}TCID_{50}$/mL) *n* positive/*n* tested (%)	0/349	0/343	2/170 (1.2)
BTV 16 (6.1 $log_{10}TCID_{50}$/mL) *n* positive/*n* tested (%)	3/728 (0.4)	0/218	1/374 (0.3)

*Venter *et al.*, 2004.

of the assay system used was 0.5 $\log_{10}TCID_{50}$ of virus per mL, this suggests that the vectors were able to be infected at very low titres indeed (i.e. <0.5 $\log_{10}TCID_{50}$ of virus per mL). However, because of differences in the various assay systems used by different researchers, direct comparisons between results are not always possible and, if made without regard to such variables, can be deceptive. However, this seems to indicate that any titre of viraemia may be sufficient to establish an infection in a proportion of biting vectors. The number infected will depend upon the virus titre in the vertebrate host, the size of the vectors' bloodmeal, their biting rate and the competence level of the specific vector population or species involved. It has recently been reported that the vaccine strains of virus used in the current study may cause a viraemia in Dorset Poll sheep that persists for up to 17 days, with peak titres in the range of 2.5–6.3 $\log_{10}TCID_{50}$/mL. Such viraemias are easily sufficient to infect most *Culicoides* vectors (Veronesi *et al.*, 2005).

In the current study, the virus titres observed indicated that the virus had replicated to high levels in at least some of the infected midges (Table 2). A concentration of live virus in the head or body of a midge higher than 2.5 $\log_{10}TCID_{50}$/insect has been postulated as an indicator of a fully disseminated infection (Jennings & Mellor, 1987) and this threshold titre was therefore used to distinguish between potentially transmissive and non-transmissive individuals. It is, however, unknown if this threshold, which was derived from a *C. sonorensis*-BTV laboratory-based model, can be used as a criterion in the interpretation of vector competence results in other, smaller, *Culicoides* spp. By contrast with the highly standardized *C. sonorensis*-BTV model, vector competence studies on field-collected *Culicoides* spp. cannot be fully controlled. *Culicoides* is a biologically highly diverse genus and thus extrapolation of vector competence data from one species to another s is not recommended (Tabachnick, 1992). Nevertheless, the results of the present study with *C. imicola* and other livestock associated *Culicoides* spp., which included the detection of virus titres up to 4 $\log_{10}TCID_{50}$/midge at 10 days post-infection, indicated that transmission of the attenuated vaccine strains of BTV by at least a proportion of infected individuals will be possible. The higher mean virus titres found in *C. bolitinos* after incubation was indicative of a higher transmission potential in this species.

In the present study no significant differences in virus recovery rates were detected in midges fed on the attenuated vaccine strains of virus before and after passage through the insect vector and *Ae. albopictus* C6/36 cell cultures (Table 4). It must, however, be noted that there is no evidence that virus passaged through mosquito cells is biologically identical (or different) to virus passaged through midges. The fact that several *Culicoides* species are competeut vectors of BTV but mosquitoes are not probably reflects differences at the gut wall level as the cells of the hind part of the midgut are where virus ingested with a bloodmeal first establishes an infection in the vector. Although such differences between vector and non-vector species or populations may be subtle, they are critical in terms of the ability of a virus to orally infect a potential vector. The results of the present study indicate that passaging of the virus through an insect-only system did not result in the selection of progeny virus populations that were even more infectious for the vectors.

The current study indicated that the oral susceptibility of South African populations of *C. imicola* and *C. bolitinos* to infection with the attenuated vaccine strains of BTV-1, -4, -9 and -16 remained similar for at least three seasons. Interseasonal stability in vector competence has also been noted for field-collected *C. sonorensis* infected with BTV-10 (Gerry *et al.*, 2001). The ability of *C. sonorensis* to support viral replication has been shown to be at least partially under genetic control (Tabachnick, 1991), which may make this trait relatively stable within particular vector populations over time.

A potential problem when using field-collected *Culicoides* from an endemic area to determine oral susceptibility for a virus is the possible presence of field viruses. However, in South Africa the prevalence of infections in field-collected vector populations, even in outbreak situations, is generally very low ($<0.06\%$) (Gerry *et al.*, 2001; Meiswinkel & Paweska, 2003). Therefore, the virus recovery rates in this study, ranging between 0.6% and 62.5%, for *C. imicola* and *C. bolitinos*, respectively, were likely the result of laboratory infection. This is supported by the fact that the identity of all the virus isolates recovered from the experimental midges was confirmed as the serotype of BTV used for infection.

Vector competence is an indication of the ability of a vector to support pathogen replication and is one of the components of vectorial capacity that is defined as the relative measure of a vector population to transmit a pathogen to a vertebrate population (Mullens, 1992). In addition to vector competence, vectorial capacity depends on biting rate, host selection, vector survival and the extrinsic incubation period of the virus. Although vector competency tests provide important information about a specific vector population, they provide little predictability about the behaviour of other populations with the same or different strains of virus (Tabachnick, 1992). It should be recognized therefore that vector species and different populations within a species vary greatly with respect to BTV susceptibility and some field populations of a vector species may be completely resistant to infection by some serotypes (Ward, 1994; Carpenter *et al.*, 2006). Several factors, including population size, infection rate, feeding rate, host preference, survival rate and age of midges determine how efficiently a *Culicoides* vector population will transmit a virus. Usually, because infection rates are so low, large numbers of vectors are required before virus transmission becomes sustainable (Muller *et al.*, 1982; Mullens *et al.*, 2004).

Given the very high abundance of the two most competent Orbivirus vectors in South Africa, *C. imicola* and *C. bolitinos* (Venter *et al.* 1996); Meiswinkel *et al.*, 2004b), the reported levels of viraemia in vaccinated animals (Dungu *et al.*, 2004a; Veronesi *et al.*, 2005) and our present results, it seems likely that transmission of BTV vaccine strains by *Culicoides* from vaccinated to unvaccinated animals may occur under field situations. Further studies, including sequence analysis of vaccine strains and field isolates of BTV, are needed for more conclusive proof of the ability of *Culicoides* spp. to vector live-attenuated vaccine strains of BTV in South Africa.

Acknowledgements

The authors acknowledge the European Commission (contract no: QLK2-2001-01722) and ARC-OVI for supporting this study.

We thank Pamela Hunter for supplying vaccine strains of BTV and Dr Baltus Erasmus for supplying the passage history of the BTV live-attenuated vaccine strains used in this study. We thank Ina Hermanides and Sandra Prinsloo for technical assistance and the OIE Reference Laboratory for Bluetongue at ARC-OVI for supporting this work. We also thank Ralph Burls for making his farm available as a collecting site and Dr Karin Kappmeier Green for constructive comments on earlier drafts of this manuscript.

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Accepted 21 May 2007

Medical and Veterinary Entomology (2007) **21**, 377–383

Virus recovery rates for wild-type and live-attenuated vaccine strains of African horse sickness virus serotype 7 in orally infected South African *Culicoides* species

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Abstract. Previously reported virus recovery rates from *Culicoides (Avaritia) imicola* Kieffer and *Culicoides (Avaritia) bolitinos* Meiswinkel (Diptera, Ceratopogonidae) orally infected with vaccine strain of African horse sickness virus serotype 7 (AHSV-7) were compared with results obtained from concurrently conducted oral infections with five recent AHSV-7 isolates from naturally infected horses from various localities in South Africa. *Culicoides* were fed sheep bloods spiked with $10^{7.6}$ $TCID_{50}$/mL of a live-attenuated vaccine strain AHSV-7, and with five field isolates in which virus titre in the bloodmeals ranged from $10^{7.1}$ to $10^{8.2}$ $TCID_{50}$/mL). After an extrinsic incubation of 10 days at 23.5°C, virus recovery rates were significantly higher in *C. imicola* (13.3%) and *C. bolitinos* (4.2%) infected with the live-attenuated virus than in midges infected with any of the field isolates. The virus recovery rates for the latter groups ranged from 0% to 9.5% for *C. imicola* and from 0% to 1.5% for *C. bolitinos*. The *C. imicola* population at Onderstepoort was significantly more susceptible to infection with AHSV-7 isolated at Onderstepoort than to the virus strains isolated from other localities. Results of this study suggest that tissue culture attenuation of AHSV-7 does not reduce its ability to orally infect competent *Culicoides* species and may even lead to enhanced replication in the vector. Furthermore, oral susceptibility in a midge population appears to vary for geographically distinct isolates of AHSV-7.

Key words. *Culicoides bolitinos*, *Culicoides imicola*, African horse sickness virus, live-attenuated vaccine, oral susceptibility, South Africa.

Introduction

African horse sickness (AHS) is a non-contagious, infectious, arthropod-borne disease of equines, caused by African horse sickness virus (AHSV), genus *Orbivirus*, Family *Reoviridae* (Coetzer & Erasmus, 1994). It is endemic in sub-Saharan Africa; however, devastating outbreaks of the disease have been recorded in India (Howell, 1963) and southern Europe (Mellor *et al.*, 1990; Mellor, 1993). With mortality exceeding 90% in susceptible horse populations, AHS is one of the most lethal viral diseases of equids (Mellor & Hamblin, 2004). Nine distinct serotypes of AHSV (AHSV 1–9) are known (McIntosh, 1958; Howell, 1962) and to be fully protected against AHSV, horses have to be immune to all serotypes (Coetzer & Erasmus, 1994). Although some promising results with recombinant vaccines have been reported (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Scanlen *et al.*, 2002), immunization with live-attenuated AHS vaccines still remains the only practical choice in the field (Sánchez-Vizcaino, 2000).

The decline in the number of AHS outbreaks in South Africa during the last few decades is partly a result of the introduction of a polyvalent AHS vaccine in 1974. Until 1990 this vaccine

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comprised two quadrivalent vaccines, one containing serotypes-1, -3, -4 and -5 and the other serotypes-2, -6, -7 and -8. Because of safety problems, the vaccine strain of AHSV-5 was discontinued in 1990 (Van Dijk, 1998). Attenuation of these viruses has usually been achieved through three initial passages in mouse brain, followed by 10 passages in baby hamster kidney-21 cells (BHK-21 cells) and, finally, by selection of avirulent large plaques in Vero cells (Erasmus, 1976). The selected plaques were passaged further in Vero cells to prepare large volumes of master seed stocks for vaccine production. Working stocks for assembling polyvalent vaccines are currently produced in BHK-21 cells (Erasmus, 1976; Paweska *et al.*, 2003).

Several studies have shown *Culicoides (Avaritia) imicola* Kieffer to be the major vector of AHSV in Africa (Venter & Sweatman, 1989; Venter & Meiswinkel, 1994; Meiswinkel, 1997; Bouayoune *et al.*, 1998). However, other *Culicoides* species may also act as competent field vectors of this virus (Boorman *et al.*, 1975; Mellor *et al.*, 1975, 1990, 2000; Wellby *et al.*, 1996; Venter *et al.*, 2000). In South Africa, a second *Avaritia* species, *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel, has been shown in the laboratory (Venter *et al.*, 2000; Paweska *et al.*, 2003) and in the field (Meiswinkel & Paweska, 2003) to be a competent vector of AHSV. *Culicoides bolitinos* has a wide distribution in southern Africa and the fact that it breeds in bovine dung (Nevill, 1968; Meiswinkel, 1989) makes this species less dependent on average annual precipitation, seasonal fluctuations in soil temperature and soil type, thereby enabling its presence in cooler areas, where it may become the most abundant species (Meiswinkel & Paweska, 2003).

Potential risks of using live-attenuated vaccines include reassortment of vaccine virus with wild-type virus strains in the mammalian hosts and/or insect vectors, virus spread by insect vectors and reversion to virulence of the vaccine strains in the insect vector (Paweska *et al.*, 2003). Data on oral susceptibility and vector competence of *Culicoides* midges for live-attenuated vaccine strains of orbiviruses are important for risk assessment, particularly when intervention with such vaccines is to be considered in regions vulnerable to virus incursions. In this context it is worth mentioning that active circulation of live attenuated bluetongue virus serotype-2 has recently been documented among unvaccinated cattle in central Italy (Ferrari *et al.*, 2005). Mouse brain (Riegler, 2002) and tissue culture (Paweska *et al.*, 2003) attenuated strains of AHSV were shown to orally infect *Culicoides*, which suggests that attenuation of this virus, either *in vivo* or *in vitro* systems does not necessarily affect its ability to infect *Culicoides* vectors. Paweska *et al.* (2003) demonstrated that *C. imicola* and *C. bolitinos* are susceptible to oral infection with most of the seven AHSV live-attenuated vaccine strains currently being used for massive annual vaccination of horses in South Africa, with the highest virus recovery rate for AHSV-7. Because of the lack of vector competence data for the non-attenuated parent vaccine strains, the extent to which the attenuation process affects the ability of AHSV to infect *Culicoides* vectors remains unknown.

With the aim of partially addressing this issue, the present study focuses on the comparison of previously reported recovery rates in *C. imicola* and *C. bolitinos* for the AHSV-7 vaccine strain with that for field isolates of AHSV-7 recovered in 1998–1999 from naturally infected horses in different regions of South Africa. This study will also provide comparative oral susceptibility in a midge population for geographically distinct isolates of AHSV-7.

Materials and methods

Insects

Adult *Culicoides* species were collected using 220-V black-light traps as described by Venter *et al.* (1998). Similar to the study of Paweska *et al.* (2003) using live-attenuated strains of AHSV, collections of midges for oral infection with field isolates of AHSV-7 were carried out during January–April 2001 at the Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI) (25°29′S, 28°11′E; 1219 m a.s.l.), and during February–May 2001 on Koeberg Farm near Clarens (28°32′S, 28°25′E; 1631 m a.s.l.) in the high-lying eastern Free State.

Virus

The identification and geographical origins of the AHSV-7 field isolates used in this study are listed in Table 1. The geographical distances between the sites varied from 20 km to 500 km. Field isolates of AHSV-7 were obtained from the Office International des Epizooties (OIE) reference centre for AHSV at the ARC-OVI and represent diagnostic submissions received from different geographical regions in South Africa during 1998 and 1999 (Table 1). Viruses were isolated by standard inoculation of equine blood or homogenized organ samples into suckling mouse brains, followed by one or two passages in BHK-21 cells. Serotyping was performed by virus neutralization assays using type-specific antisera produced in guinea pigs (House *et al.*, 1990).

Stocks of field isolates for oral infection studies were grown, titrated and stored as described previously for the vaccine strains (Paweska *et al.*, 2003). Stock virus titres expressed as $\log_{10}TCID_{50}$/mL were calculated by the method of Kärber (1931). The virus titres in the bloodmeals used for oral infection of *Culicoides* midges are shown in Table 2.

Feeding technique

Culicoides were held without access to nutrients or water for 24 h at 23.5°C and a relative humidity (RH) of 50–70% before

Table 1. Designation and origin of AHSV-7 isolates used.

Isolate designation	Origin	Year of isolation
HS23/98	Pietermaritzburg (29°40′S, 30°25′E)	1998
HS1/99	Krugerdorp (26°6′S, 27°46′E)	1999
HS3/99	Derdepoort (25°35′S, 28°21′E)	1999
HS7/99	Onderstepoort (25°39′S, 28°11′E)	1999
HS29/99	Rustenburg (25°38′S, 27°15′E)	1999

Table 2. Recovery of AHSV-7 field isolates and AHSV-7 vaccine strain from *Culicoides* after 10 days extrinsic incubation at 23.5°C.

AHSV isolate	31/62*	HS23/98	HS1/99	HS3/99	HS7/99	HS29/99
Virus titre in bloodmeal ($log_{10}TCID_{50}$/mL)	7.6	7.6	8.2	8.5	7.1	7.1
No. of feeding attempts	5	1	4	4	1	5
No. of engorged midges	959	440	975	1011	961	796
No. midges tested after incubation	487	263	383	535	445	294
Virus recovery rates in midges assayed immediately after feeding						
C. imicola	14/14† (100)‡	2/10 (20.0)	3/8 (37.5)	5/11 (45.4)	3/9 (33.3)	4/9 (44.4)
C. bolitinos	9/9 (100)		2/4 (50.0)	1/5 (20.0)		3/9 (33.3)
C. magnus	1/1 (100)		2/5 (40.0)			1/3 (33.3)
C. leucostictus			0/1 (0.0)			
C. pycnostictus						0/1 (0.0)
Virus recovery rates in midges assayed after incubation						
C. imicola	18/135 (13.3)	0/239 (0.0)	1/159 (0.6)	1/222 (0.5)	34/358 (9.5)	0/130 (0.0)
C. bolitinos	8/192 (4.2)	0/2 (0.0)	1/66 (1.5)	1/110 (0.9)	0/6 (0.0)	0/83 (0.0)
C. bedfordi	1/6 (16.7)					
C. dutoiti	1/2 (50.0)			0/3 (0.0)		
C. engubandei	0/20 (0.0)		1/1 (100)	0/1 (0.0)		
C. gulbenkiani	0/4 (0.0)		0/4 (0.0)	1/6 (16.7)		

*Paweska *et al.* (2003).
†Number positive/number tested.
‡(%).

feeding. Surviving flies were subsequently fed in batches of 250–300 for 30–45 min on defibrinated sheep blood containing one of the AHSV isolates, using a 1-day-old chicken skin membrane technique (Venter *et al.*, 1998). During feeding, lighting in the room was dimmed to ~1% daylight (~65 lux). Each blood-virus mixture was freshly prepared immediately before feeding as described by Paweska *et al.* (2003). The mixture was maintained at 35.5°C and stirred constantly during feeding. Blood-engorged females were separated out on a refrigerated chill-table and incubated for 10 days at 23.5°C. Engorged females were maintained on 5% (w/v) sucrose solution containing 500 IU penicillin, 500 μg streptomycin and 1.25 μg fungizone per mL of sucrose solution. All *Culicoides* females surviving the incubation period were sorted into species and stored individually in 1.5-mL microfuge tubes at −70°C until they could be assayed.

Field controls

In order to assess the possibility of flies having been infected with AHSV in the field, parous *C. imicola* and *C. bolitinos* females were collected regularly during the same period as midges for oral infection. Collection, age grading and attempted virus isolations from these flies were made as previously described (Meiswinkel & Paweska, 2003).

Processing of Culicoides, virological assays and statistical analysis

Flies were assayed for virus immediately after feeding on blood-virus mixtures and after 10 days of extrinsic incubation at 23.5°C as previously described by Paweska *et al.* (2003). Processing of field samples and virus isolation were performed as described by Meiswinkel & Paweska (2003). Statistical differences between experimental groups were analysed using Fisher's exact test. *P*-values of < 0.05 were used as the cut-off for statistical significance.

Results

Of 4131 *Culicoides* fed, a total of 2407 (58.3%) survived the 10-day incubation period at 23.5°C. The number of midges assayed immediately after feeding and after incubation as reflected in Table 2 was dependent on the number and species present in the field and on the survival of these species under laboratory conditions. Samples of AHSV were isolated from 31 of 61 (50.8%) *C. imicola* and from 15 of 27 (55.6%) *C. bolitinos* assayed immediately after feeding.

In *C. imicola* assayed after 10 days extrinsic incubation the highest virus recovery rate was for the attenuated vaccine strain of AHSV-7 (HS31/62) (Table 2). The virus recovery rate of the AHSV-7 attenuated strain from *C. imicola* was significantly higher ($P < 0.001$ in all cases) than those of the HS23/98 (Pietermaritzburg), HS1/99 (Krugersdorp), HS3/99 (Derdepoort) and HS29/99 (Rustenburg) isolates. There was no significant difference ($P = 0.249$) in virus recovery rate for AHSV vaccine strain (HS31/62) and HS7/99 (Onderstepoort). Two isolates, HS23/98 (Krugersdorp) and HS29/99 (Rustenburg), could not be recovered from 239 and 130 *C. imicola* individuals assayed after incubation, respectively (Table 2).

The numbers of *C. bolitinos* assayed after incubation were lower than those of *C. imicola* and three of the field isolates used could not be recovered from this species (Table 2). Similarly to *C. imicola*, the highest virus recovery rate in *C. bolitinos* was for the attenuated vaccine strain of AHSV-7 (HS31/62), but

this was not significantly different from those of HS23/98 (Pietermaritzburg) ($P = 1.000$), HS1/99 (Krugersdorp) ($P = 0.455$), HS3/99 (Derdepoort) ($P = 0.163$), HS7/99 (Onderstepoort) ($P = 1.000$) or HS29/99 (Rustenburg) ($P = 0.111$).

The virus recovery rate of the AHS-7 attenuated vaccine strain (HS31/62) was significantly higher in *C. imicola* than in *C. bolitinos* ($P = 0.003$). No significant difference in virus recovery rates from *C. imicola* and *C. bolitinos* were found for HS1/99 (Krugersdorp) ($P = 0.506$), HS3/99 (Derdepoort) ($P = 0.554$) and HS7/99 (Onderstepoort) ($P = 1.000$).

Vaccine strain was also recovered from one of six specimens of *Culicoides* (Unplaced) *bedfordi* Ingram & Macfie and from one of two *Culicoides* (*Synhelea*) *dutoiti* de Meillon assayed after incubation (Table 2). In addition, HS1/99 (Krugersdorp) was recovered from a single specimen of *Culicoides* (*Pontoculicoides*) *engubandei* de Meillon and HS3/99 (Derdepoort) from one of six *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro (Table 2). No virus could be recovered from 12 other *Culicoides* species assayed after incubation (Table 3).

All 36 pools of field-collected *C. imicola* and *C. bolitinos*, containing a total of 3600 parous females, tested negative for the presence of AHSV. This result indicated that the field infection prevalence, if present, was below 0.03% (Chiang & Reeves, 1962).

Discussion

Live-attenuated vaccines should be safe, avirulent and efficacious and should not revert to virulence in vaccinated animals or be transmitted from vaccinated animals to susceptible hosts by insect vectors. The latter criterion is very important because insect-mediated transmission of an attenuated virus from vaccinated to susceptible animals, with subsequent replication in mammalian host species, increases the possibility of reversion to virulence. The potential risk also includes vaccine virus reassortment with wild-type virus, which may generate virus strains with new virulence characteristics (Riegler *et al.*, 2000; Riegler, 2002). This study demonstrated generally higher recovery rates for AHSV-7 vaccine strain than for field isolates of the same serotype, and the fact that vaccine virus was recovered from *Culicoides* belonging to different subgenera increases safety risks associated with the use of live-attenuated vaccines.

Paweska *et al.* (2003) showed that South African *Culicoides* species are susceptible to oral infection with live-attenuated AHSV strains currently used in the production of the Onderstepoort polyvalent vaccine. These authors also demonstrated that the AHSV-7 vaccine strain in *C. imicola*, *C. bolitinos*, *C. bedfordi* and *C. dutoiti*, and the AHSV-4 vaccine strain in *C. bolitinos* replicated to a titre which, in previous studies with the closely related bluetongue virus and AHSV in *Culicoides* (*Monoculicoides*) *sonorensis* Wirth & Jones (Jennings & Mellor, 1987; Riegler, 2002), indicated fully disseminated infections and, by analogy, would therefore be sufficient for transmission by these species when feeding on susceptible equids. The results of this comprehensive study indicate a significant lower oral susceptibility of *C. imicola* to field isolates of AHSV-7 than to the attenuated AHSV-7 vaccine strain (Table 2). The attenuated AHSV-7 vaccine strain has been intensively used in the current formulation of the polyvalent vaccine for more than three decades and was shown to replicate to potentially transmissible levels in the most abundant and wide-spread, livestock-associated *Culicoides* species in South Africa, namely *C. imicola* and *C. bolitinos*. Thus, significantly lower recovery rates for field isolates representing wild-type AHSV-7 than for AHSV-7 vaccine strain might indicate that the latter had successfully co-adapted to *Culicoides* vectors over the long period of its wide and intensive use in the immunization of horses in South Africa. In this respect, it is worth mentioning that sequencing analysis of VP2 genes of AHSV-2 suggest a very close genetic relationship between the AHSV-2 isolates recovered in 2002 from clinical cases in distinct geographical areas in South Africa and the current AHSV-2 vaccine strain (Paweska *et al.*, 2003). The AHSV-2 vaccine strain represents a culture-attenuated 82/61 strain of AHS isolated in South Africa in 1961 (Erasmus, 1965).

Table 3. *Culicoides* species from which neither AHSV-7 field isolates nor AHSV-7 vaccine strain could be recovered after 10 days extrinsic incubation at 23.5°C.

AHSV isolate	31/62*	A23/98	A1/99	A3/99	A7/99	A29/99
Virus titre in bloodmeal ($log_{10}TCID_{50}$/mL)	7.6	7.6	8.2	8.5	7.1	7.1
C. coarctatus	4†		2	2		
C. enderleini	2					
C. huambensis				2		
C. leucostictus	3		1	3	1	1
C. magnus	21		8	14		1
C. neavei					1	
C. nevilli	1					
C. nivosus			1		5	
C. onderstepoortensis	2			1		
C. pycnostictus	3			6	2	
C. subschultzei			1			
C. zuluensis	4	1	1	15	7	7

*Paweska *et al.* (2003).
†Number tested.

The Imicola Complex comprises at least 12 species, of which 10 occur in Africa (Meiswinkel, 1995, 2003). Another member of this complex, *Culicoides* (*Avaritia*) *brevitarsis* Kieffer, breeds in bovine dung, as *C. bolitinos* does, and is a proven vector of bluetongue in Australasia (Muller *et al.*, 1982). Previous studies have demonstrated that *C. bolitinos* is more susceptible to oral infection with bluetongue virus and supported replication of several serotypes to significantly higher titres than *C. imicola* (Venter *et al.*, 1998; Paweska *et al.*, 2002). In the present study *C. imicola* was shown to be more susceptible than *C. bolitinos* to infection with the attenuated vaccine strain of AHS-7. Another, as yet undescribed species in the Imicola Complex (Meiswinkel, 1995) breeds in zebra, rhino and horse dung, and its close association with zebra, which are thought to be the reservoir host of AHSV (Barnard, 1993), suggests that it may be important in the epidemiology of AHSV. However, this and the other species comprising the Imicola complex are relatively scarce compared with *C. imicola* and *C. bolitinos* and are difficult to obtain in sufficient numbers for laboratory investigation.

Paweska *et al.* (2003) reported recovery of live-attenuated vaccine strains of AHSV from six experimentally infected Old World livestock-associated non-*Avaritia Culicoides* species. In the present study one or more of the AHSV-7 field isolates were also recovered from a third *Avaritia* species, namely *C. gulbenkiani*, and three non-*Avaritia* species: *C. bedfordi*, *C. dutoiti* and *C. engubandei* (Table 2). Both *C. gulbenkiani* and *C. engubandei* have been shown to feed on horses (Meiswinkel *et al.*, 2004).

The highest virus recovery rate from the Onderstepoort population of *C. imicola* of an AHSV-7 strain isolated from a naturally infected horse at Onderstepoort (Table 2) and the significant differences found in the oral susceptibility of *C. imicola* to AHSV-7 isolates originating from different geographical localities might indicate a higher fitness between the virus and *Culicoides* vectors from the same locality. This preliminary observation should be elucidated further by testing more *Culicoides* populations and a variety of isolates representing different serotypes of AHSV. The practical implication of the possible co-adaptation of endemic viruses and *Culicoides* species within geographically distinct areas is that a true assessment of vector competence and capacity might be elusive and even more difficult to study if it required some level of real-time monitoring, such as the testing of local *Culicoides* populations using currently circulating variants of orbiviruses.

All 36 pools of field-collected *C. imicola* and *C. bolitinos*, containing a total of 3600 parous female *Culicoides*, tested negative for the presence of AHSV or any other virus (Paweska *et al.*, 2003). This indicates that field infection prevalence, if present, is below 0.03% (Chiang & Reeves, 1962; Walter *et al.*, 1980). Even in outbreak situations the field infection prevalence of AHSV is very low. During outbreaks of AHSV in 1999 and 2004 in the AHS surveillance zone of the AHSV-free area in the Western Cape, field infection rates in the dominant species, *C. imicola*, were found to be 0.003% and 0.002%, respectively (Venter *et al.*, 2006). Similar low infection prevalence was found in *C. bolitinos* in an outbreak of AHS in the eastern Free State (Meiswinkel & Paweska, 2003). In this context, the superabundance of *C. imicola* during outbreaks of AHS (Venter *et al.*, 1996) usually compensates for the relatively low infection prevalence. In a countrywide survey in South Africa conducted during 1979–1985, AHS was isolated on 66 occasions from a total of 4506 *Culicoides* pools, each consisting of 100–100 000 individuals (Nevill *et al.*, 1992). As a conservative estimate, even if all the pools consisted of only 1000 individuals this would result in a field infection prevalence of less then 0.0001%. Therefore, the virus recovery rates in the range of 0.513.3% in the present study were most likely the result of laboratory infection technique.

In this study, the virus titre in the bloodmeals used for oral susceptibility studies were in the range of 7.1–8.2 $\log_{10}$TCID$_{50}$/mL; AHSV was isolated from 31 of 61 (50.8%) *C. imicola* assayed immediately after feeding (Table 2). This low rate of virus detection is not surprising given that the average size of a bloodmeal taken by *C. imicola* or *C. bolitinos* is 0.01–0.06 μL (Venter *et al.*, 2005). Based on these results, the amount of virus in the bloodmeal was insufficient to ensure that all susceptible midges would ingest virus. The infection prevalence as determined in this study may, therefore, be lower than the natural infection prevalence in these *Culicoides* species for AHSV.

In susceptible animals that are experimentally infected with wild-type AHSV, virus titre may be as high as 7.5 $\log_{10}$TCID$_{50}$/mL of blood (Scanlen *et al.*, 2002) and viraemia may last for 2–3 weeks (Paweska *et al.*, 2003). Only attenuated viruses that generate viraemia in susceptible horses below 10^3 plaque-forming units (PFU)/mL of blood are acceptable as vaccine strains (Paweska *et al.*, 2003). Although viraemia cannot be detected in most vaccinated horses, some individuals may develop viraemia lasting for 3–5 days. The current infectivity criterion is, however, based on results in a limited number of horses used for routine vaccine safety testing. Therefore, we cannot exclude the possibility that during massive vaccination some individual animals may develop higher levels of viraemia (Paweska *et al.*, 2003). Although, AHSV vaccine strains generate lower viraemia levels compared with wild-type strains of the virus, results of this study seem to indicate that the attenuation process does not decrease the ability of AHSV to replicate in vector-competent midges.

Acknowledgements

We thank the Office International des Epizooties Reference Centre for African Horse Sickness for the field isolates of African horse sickness virus used in this study and for supporting this work. We thank Ina Hermanides for technical assistance, Ralph Burls for making his farm available as a collecting site, and Alan Kemp for reading this manuscript.

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Accepted 25 September 2007

Rev. sci. tech. Off. int. Epiz., 2006, 25 (3), 1097-1109

Investigations on outbreaks of African horse sickness in the surveillance zone in South Africa

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Submitted for publication: 2 September 2005
Accepted for publication: 20 March 2006

Summary

Confirmed outbreaks of African horse sickness (AHS) occurred in the surveillance zone of the Western Cape in 1999 and 2004, both of which led to a two-year suspension on the export of horses. Light trap surveys in the outbreek areas showed that known vector competent *Culicoides* species, notably *C. imicola*, were abundant and present in numbers equal to those in the traditional AHS endemic areas. Isolations of AHS virus serotypes 1 and 7, equine encephalosis virus, and bluetongue virus from field-collected *C. imicola* in the surveillance zone demonstrated that this species was highly competent and could transmit viruses belonging to different serogroups of the *Orbivirus* genus. Molecular identification of recovered virus isolates indicated that at least two incursions of AHS into the surveillance zone had taken place in 2004. The designation of an AHS-free zone in the Western Cape remains controversial since it can be easily compromised, as evidenced by the two recent outbreaks. In light of the results reported in the present study, the policy of maintaining a large population of unvaccinated horses in the surveillance zone should be reconsidered, as it leaves them vulnerable to infection with AHS virus, which is the most pathogenic of all equine viruses.

Keywords

African horse sickness – Bluetongue – Culicoides imicola – Equine encephalosis – Field infection prevalence – Light trap survey.

Introduction

African horse sickness (AHS) is an arthropod-borne viral disease that affects all equids. The causal agent, AHS virus (AHSV), is endemic in sub-Saharan Africa and possibly Yemen; however, outbreaks do occur periodically in countries far beyond these areas (23). To date, nine distinct serotypes of AHSV have been identified (13, 17). African horse sickness is the most lethal viral disease of horses (8). It was the only equine disease included in the former List A of diseases notifiable to the World Organisation for Animal Health (OIE) (48).

African horse sickness virus has been present in Africa for many centuries. In South Africa, where no indigenous horses existed, it was first noticed in 1652 after the introduction of horses from Europe and the Far East into the Cape of Good Hope (12). The disease was frequently mentioned in the records of the Dutch East India Company, and in 1719 nearly 1,700 horses in the Cape of Good Hope succumbed to the dreaded horsesickness, also known as 'perreziekte' or 'pardeziekte' (35). The decline in the number of AHS outbreaks over the last few decades of the 20th Century, particularly in the southern areas of South Africa, is partly due to the elimination of large free-ranging populations of zebra (*Equus burchellii*), which are considered to be the natural cycling host for the virus (2, 23). Of equal importance was the introduction of a polyvalent AHS vaccine in 1974, which created a barrier of immune horses that probably impeded the southerly

spread of AHSV in South Africa. Major epidemics of AHS occur every ten to fifteen years in South Africa, and a strong link between the timing of the epidemics and the warm El Niño/Southern Oscillation has been identified (3).

The endemnicity of AHSV in southern Africa greatly disrupts the movement of horses from South Africa to Europe and the rest of the world. Traditionally, AHS was most prevalent in the northern parts of South Africa. Data from 1955 up to 1995 indicated that outbreaks of AHS occurred every summer in the northern provinces of South Africa, approximately once every five to ten years in the centre of the country, and only rarely in the Western Cape (5). Since 1950, confirmed outbreaks of AHS in the Western Cape were reported in 1967 (5), 1990 (9), 1999 (4), and 2004. It has been postulated that outbreaks of AHS in the Western Cape occurred following the transportation of viraemic horses from endemic areas (4, 5, 11).

In a six-year survey from 1979 to 1985, *Culicoides* midges were collected at 25 sites throughout South Africa and 918 virus isolates were identified from 4,506 pools of midges (26). While AHSV was isolated in 66 cases, of which 94% were from midges collected in the north of the country, it was not isolated from midges collected in the Western Cape (26).

Based on the data of Bosman *et al.* (5), an area within Cape Town was identified as AHS-free. Surrounding this declared AHS-free area is a surveillance zone and an outer protection zone. The AHS-free area is approximately 140 km² and comprises part of Metropolitan Cape Town (11). The surveillance zone has a minimum radius of 50 km (10,000 km²). The protection zone has a minimum radius of 100 km (180,000 km²) and separates the surveillance zone from the rest of South Africa (AHS-infected zone). The AHS-free area is separated from the surveillance zone by major thoroughfares, and the surveillance zone is separated from the protection zone by a river to the north and a mountain range to the east. The provincial border of the Western Cape forms the boundary between the protection and infected zones (11). These zones are managed in such a way that movements of all horses are monitored and are subject to very specific conditions (11). In addition, sentinel animals are regularly monitored for antibodies to AHSV and all mortalities in horses are investigated. Vaccination of all horses is prohibited in the AHS-free area and the surveillance zone (11).

African horse sickness virus is only transmitted when infected *Culicoides* species (Diptera: Ceratopogonidae) feed on a susceptible host, and only certain species of the *Culicoides* genus are classified as competent vectors (23). The most important vector is the Afro-Asiatic species: *Culicoides (Avaritia) imicola* Kieffer (23). The geographical distribution and seasonal incidence of AHSV is thus limited not only by the availability of the virus and susceptible vertebrate hosts but also by the presence of competent arthropod vectors. Prior to 1999, only two light trap surveys were conducted in the Stellenbosch area (situated in the Western Cape, South Africa) (25, 37, 41, 42). As part of a countrywide survey (26, 41, 42), 62 collections were made from January 1984 to November 1986 at one site (located near cattle on the Welgevallen Research Farm), and in November 1986 a more intensive survey was conducted at 22 sites in the Stellenbosch area (25). These surveys indicated that *C. imicola*, a proven vector of AHSV, was less abundant in the Stellenbosch area than in the traditional AHS-endemic areas of the country, which are characterised by summer rainfall. The November 1986 Stellenbosch survey (25) confirmed the results of the two-year Welgevallen survey (1984 to 1986) (37) and revealed that *C. (Avaritia) gulbenkiani* Caeiro, *C. (Culicoides) magnus* Colaço, *C. (Hoffmania) zuluensis* de Meillon, and *C. imicola* alternated as the dominant species in light trap collections. Data from the two-year study indicated that *C. imicola* constituted 11.3% of 145,297 midges collected in 62 collections (an average of 265 specimens were collected per trap per night) (41, 42). In the November 1986 study, *C. imicola* represented 18.4% of 33,564 *Culicoides* collected in 44 collections (an average of 141 *C. imicola* specimens were collected per night) (25). The relatively low abundance of *C. imicola* observed in these studies contrasted with the *Culicoides* population numbers in the rest of the country, especially in the traditional AHS-endemic areas, where *C. imicola* can become superabundant near livestock (21). For example, *C. imicola* represented 98% of 104,720 midges (146 collections) and 93% of 1,730,191 midges (39 collections) collected at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) (40) and the Eiland holiday resort, respectively. Both areas are located in the north of the country (42).

In 1999 and 2004, outbreaks of AHS occurred in the Stellenbosch area in the surveillance zone surrounding the declared AHS-free area. Both outbreaks led to a two-year embargo on the export of horses from South Africa and cost the industry approximately R50 million (US$ 8.2 million) per year. During the 1999 outbreak of AHS, 14 equine deaths were reported during a period of one week on seven premises as a result of infection with AHSV serotype 7 (4). In the 2004 outbreak, 16 deaths on eight premises and 16 clinical cases on a further 11 premises were reported between February and April 2004 as a result of infection with AHSV serotype 1. Because the Stellenbosch area falls within the surveillance zone of the Western Cape, none of the 8,000 equines in this area are vaccinated against AHS. However, in an effort to contain the spread of the virus during the last two outbreaks, all equines in the Stellenbosch area were vaccinated with a commercially available polyvalent AHS vaccine.

The 1999 and 2004 outbreaks of AHS in the Stellenbosch area prompted an entomological and virological investigation, the results of which are presented in this paper.

Materials and methods

Study area

The Stellenbosch area referred to in the present study is similar to the area surveyed in 1986 (25) and covers 540 km², extending from 33°40' to 34°00' S and from 18°40' to 19°00' E. The south-eastern part is mountainous, with wide, flat valleys opening into gently rolling countryside in the north-west. The Stellenbosch area is a winter rainfall region, receiving between 600 mm and 1,000 mm of rain per annum depending on the proximity to the mountains, where precipitation is higher (34). The six months from April to September receive 75% of the annual total rainfall for the region. Summers are relatively hot and temperatures below freezing and frosting are seldom recorded in the winter. At Elsenburg, where the Western Cape Department of Agriculture is located and the first horse deaths occurred in the 2004 outbreaks of AHS, the mean daily minimum temperature for the coldest month (July) is 7°C (34). The natural vegetation of the study area is classified as Coastal Rhenoster-bosveld and Macchie or Fynbos (1); however, except in protected areas, very little of this vegetation remains. Much of the region is intensively farmed, especially in areas where water is available for irrigation during the dry summer. In these areas the production of grapes and deciduous fruits is important, as is the production of kikuyu, white clover, and other types of pasture for intensive livestock farming. In areas where water availability is limited, winter wheat is planted and livestock are raised on natural pastures (25).

Culicoides collections

Culicoides species were collected from dusk to dawn on farms near livestock, mostly horses, using five 220 V black light down-draught suction light traps. Collections were made directly into phosphate buffered saline (PBS) to which 0.5% Savlon antiseptic (Clorhexidane gluconate 0.3 g/100 ml and Cetrimade 3.0 g/100 ml [Johnson & Johnson, SA]) had been added (45). Collected samples were kept at 4°C until processed for the presence of virus. During species identification, *Culicoides* were age-graded, according to the method of Dyce (10), and sorted in PBS into pools of 100 to 500 parous females per species. As there is no proof that arboviruses can be transmitted transovarially in *Culicoides* species (27), only parous females were used for virus isolation attempts.

Virus isolation from midge pools

Midge pools were washed twice in fresh PBS and then homogenised in 250 µl of chilled Eagles minimum essential medium (EMEM) containing antibiotics and an antimycotic (200 µg/ml of penicillin/streptomycin and 2.5 µg/ml of amphotericin B [Fungizone®]) using a battery-powered microtissue grinder (43). Homogenised samples were centrifuged at 14,000 × g for 10 min at 4°C and the individual supernatants were transferred into sterile 1.5 ml microtubes. The pellet was disrupted using a freeze-thaw technique and homogenised in 100 µl of EMEM. Homogenised pellet samples were centrifuged at 14,000 × g for 10 min at 4°C, the individual supernatants were added to the first supernatant, and 1,650 µl of EMEM was added. Samples were then filtered using 0.2 µm filters and 20% foetal calf serum (Delta Bioproducts, South Africa) was added. Half of the volume (1 ml) of each sample was diluted 1:2 in EMEM and 250 µl of the dilution was used to inoculate two 25 cm² plastic culture flasks containing 48 h-old monolayers of Vero cells. After one hour of incubation at 37°C on a platform shaker, inoculated cells were rinsed once with sterile PBS, overlaid with 10 ml of EMEM containing 1% foetal calf serum, and incubated at 37°C in a 5% CO_2 incubator. Inoculated cells were examined microscopically daily for 10 days post inoculation for cytopathic effects (CPE). In the absence of CPE, cultures were frozen at – 70°C, thawed, and re-passaged for two blind passages (20).

Virus identification

Viruses isolated from tissue cultures were identified by molecular methods (14, 16). Briefly, the total RNA was extracted from the tissue cultures and double stranded RNA (dsRNA) was isolated by means of differential precipitation with lithium chloride (LiCl) and used as templates for amplification by reverse transcription polymerase chain reaction (RT-PCR). The AHSV isolates were identified and serotyped using RT-PCR and reverse line blot hybridisation (16). Bluetongue virus (BTV) isolates were identified using a serogroup specific RT-PCR (14). Equine encephalosis virus (EEV) typing was done using a microtitre virus neutralisation procedure.

Sequence analysis

Full-length segment 2 AHSV DNA was cloned and sequenced after RT-PCR amplification from isolated dsRNA as described by Koekemoer *et al.* (15). This was done on the AHSV serotype 7 midge pool isolate from one of the collection sites in the 2004 survey and on three other serotype 7 viruses isolated from field cases sent to the OIE Reference Centre for AHS at OVI. Partial nucleotide sequences of genome segment 2 were aligned using the program CLUSTAL X (36), and the aligned sequences were

used to construct a phylogenetic tree by means of a UPGMA (unweighted pair-group method using arithmetic averages) computer algorithm.

Statistical analyses

Fisher's Exact and Kruskal-Wallis tests (Nonparametric ANOVA [Analysis of Variance]) were used to interpret statistical data.

Results

Light trap collections

The identification of the *Culicoides* species recovered from black light traps during outbreaks of AHS in the Stellenbosch area in March to April 1999 and April 2004 are shown in Table I and II respectively. In Table III the 1999 and 2004 light trap data are compared to a light trap survey done in 1986.

During the 1999 survey, a total of 212,199 *Culicoides* midges, belonging to 13 species, were collected from 26 March to 29 April 1999 in 38 light trap collections at 15 sites. Depending on the prevailing weather conditions on the night of collection, the number of hosts present, and the location of the light trap, the number of *Culicoides* midges collected per light trap per night ranged from 5 to 75,116 (average = 5,584) (Table I). The proven AHSV vector, *C. imicola*, representing 96% of the total number of midges collected, was the only species identified at all 15 collection sites (Table III). Its representation varied from 43.2 to 98.5% at the various sites and was the dominant *Culicoides* species at all 15 sites sampled (Table I). The second most abundant species, *C. zuluensis*, was collected at 14 of the 15 sites sampled and represented 1.7% of the *Culicoides* specimens collected. *Culicoides bolitinos* was found at 13 of the 15 sites sampled and was the third most abundant species, representing 1.1% of all *Culicoides* specimens collected. A further ten *Culicoides* species, each representing less than 1%, were also collected (Tables I & III).

During the 2004 survey, a total of 187,521 *Culicoides* midges, belonging to 15 *Culicoides* species, were collected in 39 light trap collections made from 1 March to 26 March 2004 at 16 sites. The number of midges collected per trap per night ranged from 1 to

Table I
Identification of *Culicoides* species recovered from black light traps during an outbreak of African horse sickness in the Stellenbosch area, southwestern Western Cape, South Africa, in March and April 1999 (percentages)

Collection site	Linquenda	Thistledown	Stellen Kloof	Remhoogte	Spier	Avontuur	Varswater	Maties Ryskool	Troughend	Elsenburg	Klein Bosch	Sandringham	Rosendal	Digteby	Glenconner
Collection date	27/3-20/4	22/4	29/3-14/4	26/3	30/3-21/4	3-30/4	23/4	28-30/3	2/4	22/4	15/4	24/4	26/3-14/4	31/3	29/4
Number of collections made	3	1	2	1	4	4	1	2	1	1	1	1	14	1	1
C. imicola	98.52	97.96	97.01	84.88	96.67	89.28	96.93	97.62	99.02	92.29	99.50	84.00	44.17	97.5	43.24
C. zuluensis	0.37	1.84	1.00	7.16	1.23	6.11	1.65	0.02	0.20	2.57	0.45	12.00	17.57		5.41
C. bolitinos	1.08	0.07	0.40	0.75	1.76	1.00	0.11	2.10	0.49	3.04		1.71	0.25	2.5	
C. magnus	<0.01	0.07	0.10	7.11	0.32	3.37			0.29	0.76		1.71	2.74		
C. leucostictus	<0.01		1.10			0.04	1.31	0.13					23.93		32.43
C. pycnostictus			0.30			0.03					0.06	0.29	8.22		18.92
C. gulbenkiani	0.02	0.07	0.02	0.05	0.02	0.03		0.13		1.33		0.29	0.12		
C. nivosus				0.05		0.03							2.31		
C. nr *angolensis*	<0.01		0.07			0.06							0.44		
C. exspectator						0.04									
C. engubandei													0.12		
C. coarctatus													0.06		
C. sp. *107*													0.06		
Total	**107,913**	**10,633**	**13,667**	**8,044**	**25,402**	**24,506**	**4,429**	**8,614**	**3,066**	**2,102**	**1,791**	**350**	**1,605**	**40**	**37**
Average catch size	35,971	10,633	6,833.5	8,044	6,350.5	6,126.5	4,429	4,307	3,066	2,102	1,791	350	114.61	40	37
Range of catch size	2,157-75,116	10,633	5,557-8,110	8,044	294-21,740	58-11,540	4,429	654-7,960	3,066	2,102	1,791	350	5-358	40	37

Table II
Identification of *Culicoides* species recovered from black light traps during an outbreak of African horse sickness in the Stellenbosch area, southwestern Western Cape, South Africa, in March 2004 (percentages)

Collection site	Linquenda	Spier	Rosendal	Natte Valeij	Del Vera	Elsenburg	Vredenheim	Dakari	Dalewood	Langeberg	Chilanga	Paarl diamond	Delmerdo (Ashire)	Through end	Ivan Stark	Staedfast farm
Collection date	**1/3**	**1-26/3**	**2/3**	**3/3**	**3-30/3**	**1-26/3**	**18-19/3**	**1/3**	**2/3**	**3/3**	**11-15/3**	**4/3**	**4/3**	**2/3**	**4/3**	**4/3**
Number of collections made	**1**	**5**	**1**	**1**	**6**	**10**	**2**	**1**	**1**	**1**	**5**	**1**	**1**	**1**	**1**	**1**
C. imicola	99.07	99.22	75.49	96.33	98.44	77.61	45.61	79.44	54.99	94.68	79.23	92.31	91.43	94.44	61.54	100
C. zuluensis	0.48	0.29	17.76	2.01	0.52	13.11	43.06	12.07	41.71	2.88	0.24	3.85	5.71	5.56		
C. bolitinos	0.38	0.27	3.10	0.45	0.78	8.42	6.74	1.16	0.21	0.78	0.35	1.92			30.77	
C. magnus	0.02	0.13	3.60	0.80	0.14	0.64	1.43	1.74	2.99	1.11			2.86			
C. leucostictus		0.02		0.15	0.01	0.02	1.18			0.11	16.86					
C. gulbenkiani	0.02					0.17	1.04	5.31								
C. pycnostictus			0.05	0.19		0.03	0.07			0.33	2.89	1.92			7.69	
C. glabripennis	0.02	0.08														
C. nivosus					0.07	0.01	0.82		0.10		0.17					
C. onderstepoortensis					0.08				0.29			0.03				
C. milnei	0.02															
C. neavei										0.11	0.10					
C. nr angolensis											0.07					
C. similis							0.04									
C. engubandei											0.03					
Total	**25,210**	**110,842**	**3,998**	**2,641**	**14,517**	**21,623**	**2,789**	**1,036**	**971**	**902**	**2,870**	**52**	**35**	**18**	**13**	**11**
Average catch size	25,210	22,168.4	3,998	2,641	2,419.5	2,162.3	1394.5	1,036	971	902	574	52	35	18	13	11
Range of catch size	25,210	48-63,260	3,998	2,641	1-14,465	1-16,887	1,100-1,689	1,036	971	902	166-1,029	52	35	18	13	11

63,260 (average = 4,808) (Table II). As in the 1999 survey, *C. imicola*, representing 94.6% of all *Culicoides* specimens collected, was the dominant species at all sites (Tables II & III). It was also the most widespread, as it was the only *Culicoides* species that was present at all of the sites sampled. As in the 1999 survey, the second and third most dominant species collected were *C. zuluensis* (3.1%) and *C. bolitinos* (1.4%), respectively. In the 2004 survey, *C. zuluensis* and *C. bolitinos* were collected at 14 and 13 of the 16 sites sampled, respectively (Table III). A further 12 *Culicoides* species, each representing less than 1% of the *Culicoides* specimens, were collected (Tables II & III).

Culicoides magnus and *C. gulbenkiani*, shown to be the co-dominant species in the 1986 survey (25), represented less than 1% of all *Culicoides* specimens collected in both the 1999 and 2004 surveys (Table III). In all three surveys, *C. imicola* was found to be widely distributed in the area and was present at all of the sites sampled. However, in the 1999 and 2004 surveys *C. imicola* was the dominant species at all of the sites sampled, which was significantly different ($P < 0.001$) from the 1986 survey, where it was dominant at only five of the 22 sites sampled (Table III). While there was no significant difference ($P > 0.05$) in the average number of *C. imicola* collected at each site between the 1999 and 2004 surveys, significantly lower numbers of *C. imicola* were collected per site in the 1986 survey than in either of the 1999 ($P < 0.001$) or 2004 ($P < 0.05$) surveys.

Similarly, there were no significant differences ($P > 0.05$) in the average numbers of *C. magnus* or *C. pycnostictus* collected at each site in the 1999 and 2004 surveys. Significantly higher numbers of *C. magnus* were, however, collected per site in the 1986 survey than in either of the 1999 ($P < 0.001$) or 2004 ($P < 0.01$) surveys. In addition, significantly higher numbers of *C. pycnostictus* were collected per site in the 1986 survey than in either of the 1999 ($P < 0.001$) or 2004 ($P < 0.001$) surveys.

The average number of *C. bolitinos* ($P = 0.275$) and *C. zuluensis* ($P = 0.237$) collected at each of the sites did not differ significantly between the three surveys.

Table III
Comparison of black light trap collections made in 1986, 1999, and 2004 in the Stellenbosch area, southwestern Western Cape, South Africa

Collection date	November 1986[a]			March-April 1999			March 2004		
Number of sites sampled (number of collections made)	22 (44)			15 (38)			16 (39)		
Culicoides species	Number of sites present (number of sites dominant)	Number collected (% of total number collected)	Average number per collection	Number of sites present (number of sites dominant)	Number collected (% of total number collected)	Average number per collection	Number of sites present (number of sites dominant)	Number collected (% of total number collected)	Average number per collection
C. imicola	22 (5)	6,186 (18.43)	140.6	15 (15)	203,774 (96.03)	5,362.5	16 (16)	177,455 (94.63)	4,550.1
C. zuluensis	22 (1)	4,122 (12.28)	93.7	14	3,588 (1.69)	94.4	14	5,878 (3.13)	150.7
C. bolitinos	22 (2)	5,018 (14.95)	114.0	13	2,258 (1.06)	59.4	13	2,685 (1.43)	68.8
C. magnus	22 (6)	8,376 (24.96)	190.4	10	1,576 (0.74)	41.5	11	571 (0.30)	14.6
C. leucostictus	21 (2)	3,750 (11.17)	85.2	6	627 (0.30)	16.5	7	547 (0.29)	14.0
C. gulbenkiani	19 (2)	2,793 (8.32)	63.5	10	90 (0.04)	2.4	4	126 (0.07)	3.2
C. pycnostictus	22 (3)	1,990 (5.93)	45.2	5	190 (0.09)	5.0	8	103 (0.05)	2.6
C. glabripennis							2	97 (0.05)	2.5
C. nivosus	19 (1)	736 (2.19)	16.7	3	49 (0.02)	1.3	5	41 (0.02)	1.1
C. nr *angolensis*				4	33 (0.02)	0.9			
C. onderstepoortensis	2	27 (0.08)	0.6				3	6 (<0.01)	0.2
C. exspectator	4	43 (0.13)	1.0	1	10 (<0.01)	0.3			
C. milnei							1	5 (<0.01)	0.1
C. neavei	3	6 (0.02)	0.1				2	4 (<0.01)	0.1
C. angolensis	5	60 (0.18)	1.4				1	1 (<0.01)	0.03
C. similis	4	28 (0.08)	0.6				1	1 (<0.01)	0.03
C. engubandei				1	2 (<0.01)	0.10	1	1 (<0.01)	0.03
C. coarctatus[b]	1	3 (0.01)	0.1	1	1 (<0.01)	0.03			
C. brucei	1	2 (0.01)	0.05						
C. accraensis	1	10 (0.03)	0.2						
C. olysiageri	3	12 (0.04)	0.3						
C. nevilli	2	16 (0.05)	0.4						
C. huambonsis	14	338 (1.01)	7.7						
C. subschultzei	4	21 (0.06)	0.50						
C. enderleini	1	1 (<0.01)	0.02						
C. sp. 107				1	1 (<0.01)	0.03			
Other	8	26 (0.08)	0.6						
Total		**33,564**			**212,199**			**187,521**	

a) *Source:* Nevill *et al.*, 1988 (25)
b) identified as *C. confusus* in the survey by Nevill *et al.*, 1988 (25)

Culicoides bolitinos was collected at all 22 sites sampled in 1986 and was the dominant species at two of the sites. This was, however, not significantly different from either the 1999 (P = 0.158) or the 2004 survey (P = 0.066), when *C. bolitinos* was collected at 13 of 15 and 13 of 16 sites, respectively.

Field infection prevalence

To determine the field infection prevalence in *Culicoides* species collected during the 1999 outbreak of AHS, 331 pools consisting of 100 parous *C. imicola* females and three pools consisting of 500 parous *C. imicola* females were analysed for the presence of virus (Table IV). While EEV serotype 1 was isolated from 13 of the pools, AHSV serotype 7 was only isolated once (Table IV). Field infection prevalence in *C. imicola* was calculated, using the method of Walter *et al.* (46), to be 0.003% for AHSV and 0.038% for EEV. Based on the field infection prevalence of the 1999 survey, the pool size for *C. imicola* was increased to 500 specimens in the 2004 survey. The number of pools

Table IV
Virus isolation results from midge pools collected with black light traps during the 1999 and 2004 outbreaks of African horse sickness in the Stellenbosch area, southwestern Western Cape, South Africa

Farm name	Collection date	*Culicoides* spp.	Pool size	Number of pools tested	Viruses isolated
1999					
Avontuur	2/4	*C. imicola*	100	46	
Avontuur	4/4	*C. imicola*	100	10	4 × EEV 1
Maties Ryskool	28/3	*C. imicola*	100	20	2 × EEV 1
Remhoogte	25/3	*C. imicola*	100	30	
Rosendale	25/3	*C. imicola*	100	1	
Spier	14/4	*C. imicola*	100	33	
Spier	29/3	*C. imicola*	100	49	
Stellenkloof	28/3	*C. imicola*	100	29	2 × EEV 1
Troughend	2/4	*C. imicola*	100	6	
Linquenda	27/3	*C. imicola*	100	107	4 × EEV-1
Linquenda	27/3	*C. imicola*	500	3	1 × EEV-1
					1 × AHSV-7
Total			**1,500**	**334**	
2004					
Elsenburg	3/3	*C. imicola*	500	7	2 × AHSV-1
Elsenburg	3/3	*C. bolitinos*	191	1	
Elsenburg	3/3	*C. imicola*	306	1	
Linquenda	1/3/	*C. imicola*	500	18	1 × BTV
Del Vera	3/3	*C. imicola*	500	6	
Rosendal	2/3	*C. imicola*	500	1	
Rosendal	2/3	*C. imicola*	412	1	
Natte Valeij	3/3	*C. imicola*	500	1	
Dalewood	2/3	*C. imicola*	185	1	
Natte Valeij	3/3	*C. imicola*	109	1	
Dakari	1/3	*C. imicola*	259	1	
Elsenburg	4/3	*C. imicola*	135	1	
Elsenburg	3/3	*C. imicola*	500	1	
Elsenburg	2/3	*C. imicola*	105	1	
Vredenheim	18/3	*C. imicola*	500	1	
Chilanga	18/3	*C. imicola*	145	1	
Elsenburg	18/3	*C. imicola*	121	1	
Elsenburg	11/3	*C. imicola*	375	1	
Elsenburg	11/3	*C. bolitinos*	176	1	
Vredenheim	19/3	*C. imicola*	172	1	
Chilanga	14/3	*C. imicola*	129	1	
Spier	24/3	*C. imicola*	500	3	
Spier	24/3	*C. imicola*	135	1	
Spier	25/3	*C. imicola*	500	18	
Spier	26/3	*C. imicola*	500	26	1 × AHSV-7
Total			**7,955**	**97**	

BTV: bluetongue virus
AHSV: African horse sickness virus
EEV: equine encephalosis virus

tested and the pool sizes for all species are given in Table IV. African horse sickness virus serotype 1 was isolated from two of the pools. African horse sickness virus serotype 7 and BTV were each isolated from one of the pools (Table IV). The field infection prevalence in *C. imicola* during the 2004 outbreak was calculated to be 0.005% for AHS serotype 1 and 0.002% for AHS serotype 7 and BTV.

Sequence analysis

Sequence analyses were carried out to determine whether the AHSV serotype 7 that was isolated from one of the midge pools from Stellenbosch in 2004 was genetically related to the virus topotype that caused the outbreak of AHSV serotype 7 in the same area in 1999. During an earlier study of the 1999 outbreak (15), three isolates from infected horses and one from a midge pool were used to sequence part of genome segment 2 (VP2-gene). The segments from all of the isolates were found to have identical nucleotide sequences (Genebank accession numbers: AY159941, AY159942, AY159940, and AY159944). A full-length genome segment 2 clone of the AHSV 7 midge pool isolate from the 2004 study (MP84/04) was used to sequence the corresponding area of the virus genome (Genebank accession number: DQ118703). The same process was repeated for three AHSV serotype 7 isolates from cases of the disease in horses from Gauteng (HS34/04, Genebank accession number: DQ118704), the North-West (HS56/04, Genebank accession number: DQ118705), and the Free State (HS57/04, Genebank accession number: DQ118706) provinces.

After a comparison of the 2004 sequence data with that from 1999, it was revealed that the nucleotide sequence of the AHSV serotype 7 midge isolate obtained in 2004 had only 95.3% identity to that of the topotype that caused the 1999 Stellenbosch outbreak in the Western Cape. The three 2004 field isolate viruses from the North-West, Gauteng, and Free State provinces all had identical nucleotide sequences over the area of interest, and the sequence showed 100% nucleic acid identity with the sequence of the midge pool isolate obtained from Stellenbosch during the same season. These relationships are graphically illustrated by the phylogenetic tree in Figure 1.

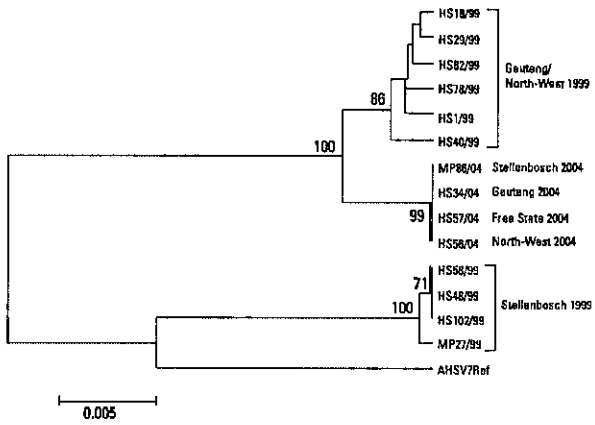

Fig. 1
UPGMA (Unweighted pair-group method using arithmetic averages) tree constructed using nucleotide sequence data from genome segment 2 (VP2-gene) of African horse sickness virus (AHSV) 7
Bootstrap confidence levels are indicated on the major nodes. The tree shows the genetic relationships between AHSV 7 isolates from the 1999 and 2004 outbreaks in South Africa. The 2004 Stellenbosch midge pool isolate (MP86/04) is grouped in close relation to the viruses isolated during the same season in the northern parts of South Africa and is clearly distinct from the group containing the viruses isolated during the previous major AHSV 7 outbreak in Stellenbosch in 1999

Discussion

Several studies have shown that the major vector of AHSV in Africa is *C. imicola* (6, 19, 38, 39). Until recently, this species was also considered the only *Culicoides* species involved in the transmission of AHSV. However, a second African species, *C. bolitinos*, which was the third most abundant *Culicoides* species in the 1999 and 2004 surveys, has been shown in the laboratory (44) and in the field (20) to be a competent vector of AHSV. *Culicoides bolitinos* has a wide distribution in southern Africa (18) and, like *C. imicola*, has also been implicated as a vector of EEV (20, 30), epizootic haemorrhagic disease of deer virus (31), and BTV (28, 43). The fact that *C. bolitinos* breeds in bovine dung (18, 24) makes this species less dependent on average annual precipitation and soil type for survival and reproduction and facilitates the penetration of the species into cooler highland areas where it is particularly abundant and *C. imicola* is less common (20, 38, 39).

The fact that AHSV, BTV, and EEV could be isolated from field-collected *C. imicola* in the present study confirms that vector competent populations of this species are present in the Stellenbosch area. While only AHSV serotype 1 was isolated from sick and dead horses as well as from field-collected midges during the 2004 outbreak, AHSV serotype 7 was only isolated from field collected *C. imicola* (Table IV).

The origin of the AHSV 7 isolate was of interest as there had previously been a major outbreak of this serotype in the Stellenbosch area (4). Genetic characterisation was used to determine whether the AHSV 7 isolate was a remnant of the previous Stellenbosch epizootic or was

a new introduction to the area. Genome segment 2 sequence data from AHSV 7 horse and midge isolates from the 1999 Stellenbosch outbreak was available from a previous study (15) and was used as a reference. Sequence data from a corresponding part of genome segment 2 indicated that there was a clear distinction between the AHSV 7 isolated from a midge pool during this study (MP84/04) and the AHSV 7 topotype that caused the 1999 outbreak in the same area (Fig. 1). However, when compared to AHS viruses circulating during the same season in the northern parts of South Africa, the AHSV 7 isolate from the present study showed 100% sequence identity. This finding, therefore, eliminated the possibility that the AHSV 7 isolate from 2004 was a carry-over from the 1999 outbreak and implied that the virus was a new introduction from the traditional AHS endemic area(s) of the country.

Because the Stellenbosch area falls within the AHS surveillance zone in the Western Cape, none of the 8,000 equines in this entire area should be vaccinated against AHS (11). However, in order to contain the 1999 and 2004 outbreaks of AHS in this area, all equines were vaccinated with a commercially available polyvalent vaccine. It has been shown that various species of *Culicoides* can replicate live-attenuated serotype specific vaccine strains that are currently used for the production of the commercial AHS polyvalent vaccine in South Africa (29). However, comparison of the nucleotide sequence of part of the genome segment 2 of the 2004 AHSV serotype 7 isolate with the sequence from the same segment from the AHSV serotype 7 vaccine strain revealed genetic differences which seemed to rule out the possibility that the vaccine strain was isolated from the midge pool (data not shown).

The 2004 results indicated that in addition to AHS serotype 1, which caused deaths in horses, AHS serotype 7, which was not detected in horses, was also present in the Stellenbosch area during this time.

Incursions of AHSV can be caused via the movement of viraemic horses from infected zones or via infected midges. Except for a single mountain range, the borders of the various zones are mostly administrative and do not safeguard the surveillance area from the movement of *Culicoides* midges, especially along the coast. There is compelling evidence that *Culicoides* can be transported by prevailing winds for hundreds of kilometres (33). It is, however, also possible that the midges can be dispersed with livestock (21). As little is known about reservoir hosts in the surveillance zone and adjoining areas, it is not possible to rule out the possibility that infected midges will enter or are already present in these areas. Considering these uncertainties, coupled with the relatively high abundance of *Culicoides* species in the surveillance zone, it is advisable that susceptible horses be protected against AHSV at all times.

The host preference of *C. imicola* for larger mammals is not restricted to horses, and taking into account the relatively low number of viraemic horses in the outbreak area and the number and variety of other mammals that the midges can feed upon, the low infection prevalence (0.003% for AHSV and 0.038% for EEV) detected in field-collected midges is not unexpected. Low infection prevalence in an insect vector is typical of an arbovirus outbreak (7, 46). However, the superabundance of *C. imicola*, as determined by light trap collection, compensates for the relatively low infection prevalence. The present abundance of midges in this area is sufficient to successfully transmit AHSV.

It was found that numbers of *Culicoides* species, especially *C. imicola*, collected during the 1999 and 2004 surveys exceeded population numbers in the traditional AHS-endemic areas during the same period. Since there is no evidence that AHSV is transmitted transovarially by the vectors, persistence of the virus is only possible in areas where active adult vectors are present throughout the year. For example, *C. imicola* can be collected in low numbers throughout winter at Onderstepoort, which is an area that experiences frost (42). Stellenbosch has a frost-free winter, so it is likely that *Culicoides* adults are present in this area during the winter (42). If a suitable reservoir host for AHSV is present it will, therefore, be possible for AHSV to overwinter in the Stellenbosch area. To ensure that the transmission cycle of AHSV is successfully broken, vector-free periods must be longer than the maximum period that circulating virus is found in the local susceptible vertebrate population following infection (22). Regarding the transovarial transmission of viruses in *Culicoides* species, it is worthwhile mentioning recent work showing that it is possible to detect BTV, a closely related *Orbivirus* in the family *Reoviridae* nucleic acid by nested RT-PCR in *C. sonorensis* larvae (47). The published data also suggest that BTV may not require abundant expression of the outer coat genes, used for viral detection, to persist in the insect vector and that this could explain the low rate of isolation of the virus from insects (47).

The distribution of AHSV is largely controlled by the abundance, prevalence, and seasonal incidence of certain species of *Culicoides* biting midges. The results of the 1999 and 2004 surveys conducted in the months of March and April on *Culicoides* species composition and abundance, indicated that the abundance of the proven AHSV vector, *C. imicola*, was up to 400 times more than the abundance reported in the November 1986 (25) survey or the two-year survey conducted from 1984 to 1986 (42). Because of seasonal variation and variation in climatic conditions between years, coupled with environmental factors on the night of collection at each site, direct comparison between these data sets is not entirely possible and conclusions must be made cautiously. It can, however, be pointed out that the maximum catch size for *C. imicola* during the two-year Welgevallen survey (1984 to 1986)

was 5,540 specimens (representing only 36.6% of the more than 15,000 midges collected) collected in April 1986. In contrast to the April 1986 data, *C. imicola* was collected in very low numbers in April 1985 (5.6% of a total of 1,632 midges collected) and April 1984 (2.5% of a total of 1,997 midges collected) at Welgevallen. The maximum number of *C. imicola* collected in the November 1986 survey (25) was 1,123 specimens (60.3% of a total of 1,863 midges collected).

During the 1999 and 2004 surveys, *C. imicola* was superabundant and the most dominant *Culicoides* species in the Stellenbosch area, indicating the important role of *Culicoides* species in the transmission of AHSV to this area. The apparent increase in *Culicoides* population numbers in comparison to previously published data for the Stellenbosch area coincided with the northward expansion of *C. imicola* into many areas of Europe, which was attributed to a corresponding change in climate in the region (32). Bluetongue virus, also transmitted by *C. imicola* and closely related to AHSV, has already entered many parts of Europe and is causing unprecedented outbreaks of disease in ruminants, suggesting that the same situation may occur with AHSV in equids (23). The abundance of most of the other *Culicoides* species collected in the present study did not change significantly from previous collections made in the Stellenbosch area. To determine if the high numbers of *C. imicola* collected in the 1999 and 2004 surveys is a true reflection of the status of the vector, or rather the result of yet undefined factors, and to confirm the absence of vector-free periods, it will be necessary to conduct a more intensive and seasonal *Culicoides* survey in this area.

Entomological and virological results presented for the Stellenbosch area in the present study demonstrated that the designation of the AHS-free zone in the south-west of the Western Cape remains controversial. The current veterinary policy of maintaining large populations of unvaccinated horses in the surveillance zone is of concern since it leaves horses vulnerable to infection with the most deadly of all equine viruses and, as evidenced by the 1999 and 2004 outbreaks, this zone can easily be compromised. It is, therefore, recommended that geographical boundaries of the zoning system for AHS and the present vaccination policies for equids residing within the various zones be reconsidered.

Acknowledgements

We thank Ina Hermanides, Karien Labuschagne, and Isabel Wright for technical assistance and the OIE Reference Laboratory for AHS at the ARC-OVI for supporting this work. We thank the Equine Research Center at the Faculty of Veterinary Science, University of Pretoria, for support during the 1999 survey and personnel from the Western Cape Department of Agriculture at Elsenburg, especially Dawid Visser and Marna Sinclair, for their support during the 2004 outbreak. We are grateful to all the horse owners and farmers in the Stellenbosch area who cooperated in this study. We thank Karin Kappmeier Green and Rudy Meiswinkel for helpful comments on the drafts of this manuscript.

■

Enquête sur les foyers de peste équine survenus dans la zone de surveillance en Afrique du Sud

G.J. Venter, J.J.O. Koekemoer & J.T. Paweska

Résumé

Deux foyers de peste équine ont été confirmés dans la zone de surveillance de la province du Cap occidental, respectivement en 1999 et en 2004, imposant à chaque fois une interruption des exportations de chevaux pendant deux ans. Une enquête utilisant la technique du piégeage lumineux dans les zones des foyers a mis en évidence que les espèces vectrices compétentes de ***Culicoides***, notamment ***C. imicola***, y étaient aussi abondantes que dans les zones où la peste équine est historiquement endémique. Les sérotypes 1 et 7 du virus de la peste équine, le virus de l'encéphalose équine et le virus de la fièvre catarrhale ovine ont été isolés à partir d'échantillons du vecteur ***C. imicola*** récoltés sur le terrain, indiquant qu'il s'agit d'une espèce compétente susceptible de transmettre des virus appartenant à plusieurs sérogroupes du genre ***Orbivirus***. La caractérisation

moléculaire des isolats viraux a révélé que le virus de la peste équine a fait au moins deux incursions dans la zone de surveillance en 2004. La désignation d'une zone indemne de peste équine dans la province du Cap occidental reste sujette à discussion en raison de la fragilité de ce statut, comme le montrent les deux récents foyers. À la lumière des résultats de cette enquête, il conviendra de réexaminer le bien-fondé de la stratégie consistant à garder une importante population de chevaux non vaccinés dans la zone de surveillance, car elle les laisse vulnérables face au virus de la peste équine, le plus pathogène de tous les virus affectant les chevaux.

Mots-clés
Culicoides imicola – Encéphalose équine – Enquête par piégeage lumineux – Fièvre catarrhale ovine – Peste équine – Prévalence de l'infection sur le terrain.

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Investigaciones sobre brotes de peste equina en la zona de vigilancia de Sudáfrica

G.J. Venter, J.J.O. Koekemoer & J.T. Paweska

Resumen
En 1999 y 2004, en la zona de vigilancia del Cabo Occidental, se produjeron sendos brotes confirmados de peste equina que obligaron a suspender durante dos años las exportaciones de caballos. Gracias a un estudio con trampas luminosas colocadas en las zonas afectadas por los brotes se descubrió que las especies que, según se sabe, son competentes con el vector, esto es, las del género *Culicoides*, y en especial *C. imicola*, eran allí tan abundantes como en zonas donde la enfermedad es tradicionalmente endémica. El aislamiento de los serotipos 1 y 7 de la peste equina y los virus de la encefalosis equina y la lengua azul en ejemplares de *C. imicola* capturados en la zona de vigilancia demostró que esta especie era muy competente y podía transmitir virus pertenecientes a distintos serogrupos del género *Orbivirus*. El análisis molecular de las muestras víricas obtenidas puso de manifiesto que en 2004 se habían producido al menos dos incursiones distintas del virus de la peste equina en la zona de vigilancia. La designación de una zona libre de la enfermedad en el Cabo Occidental no deja de suscitar controversia por la facilidad con que puede verse expuesta, como demuestran esos dos brotes. En vista de los resultados descritos en el presente estudio, convendría recapacitar sobre la política seguida hasta ahora de mantener a una gran población de caballos no vacunados en la zona de vigilancia, porque ello los hace vulnerables a la infección por el virus de la peste equina, que es el más patógeno de todos los virus equinos.

Palabras clave
Culicoides imicola – Encefalosis equina – Estudio con trampas luminosas – Lengua azul – Peste equina – Prevalencia infecciosa sobre el terreno.

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