VARIATION IN AFRICAN HORSE SICKNESS VIRUS
AND ITS EFFECT ON THE VECTOR COMPETENCE OF
CULICOIDES BITING MIDGES

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Many thanks also go to my family, who must have been glad to be far away during my write-up, and all my friends, who were not so lucky but suffered in silence.
II. Abstract

African horse sickness virus (AHSV, genus Orbivirus, family Reoviridae) possesses a genome of 10 double-stranded RNA segments, causes the often fatal equine disease African horse sickness (AHS) and is transmitted by haematophagous Culicoides midges. AHSV segment 10 codes for the nonstructural membrane proteins NS3 and NS3a. NS3 has been implicated in the process of virus release from infected cells. Its isolates show sequence variation of up to 36% and cluster into three distinct groups, while the homologous NS3 of the Orbivirus type species bluetongue virus varies by only 7%. Reassortant viruses, differing only in genome segment 10, were used in this work to show that variation in this segment can affect virus dissemination within the midgut and from the midgut into the haemocoel of C. sonorensis. This demonstrates an effect of variation in AHSV NS3 on model vector competence. The significance of this result is underlined by the effect of variation in AHSV segment 10 on oral infection rates in the field vector C. imicola. Wild-caught C. imicola and C. bolitinos were also shown to become infected with heavily attenuated AHSV strains. This indicates that the use of live attenuated AHSV vaccines may contribute to virus spread, reassortment and reversion to virulence. The results of vector competence studies with two C. sonorensis colonies, which had previously been selected for or against vector competence for AHSV, show that the midgut infection barrier of the insect is defined by both the insect and the virus. Conversely, virus ability to overcome the midgut escape barrier was mainly virus-defined and did not depend on insect genotype. A technique was developed for identifying infected insect midguts by immunofluorescent microscopy. This was applied to show that C. sonorensis (of low oral susceptibility) replicate AHSV in their midgut when infected from the abluminal side, while midgut cells of the non-vector C. nubeculosus appeared to be unable to replicate the virus. Variation in genome segment 10 affected virus dissemination within the midgut of the vector-competent colony of C. sonorensis only. It is concluded that vector competence may depend on insect interaction with NS3/3a. This could be exploited to identify the genetically determined difference between vector-competent and non-competent colonies and appropriate experiments are suggested. Sequencing of genome segment 10 of the two reassortant viruses identified differences in predicted NS3 membrane configurations and variation in their potential membrane anchoring motifs. These differences could affect insect vector competence and suitable future experiments are suggested.
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<th>Description</th>
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<tbody>
<tr>
<td>$\chi^2$</td>
<td>Chi-Square</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>a.s.l.</td>
<td>Above sea level</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid(s)</td>
</tr>
<tr>
<td>AHS</td>
<td>African horse sickness</td>
</tr>
<tr>
<td>AHHSV</td>
<td>African horse sickness virus</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BT</td>
<td>Bluetongue</td>
</tr>
<tr>
<td>BTV</td>
<td>Bluetongue virus</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>C.</td>
<td>Culicoides</td>
</tr>
<tr>
<td>Cap</td>
<td>Capping enzyme</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>d.f.</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DB</td>
<td>Dissemination barrier</td>
</tr>
<tr>
<td>DI</td>
<td>Disseminated infection</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post infection</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>E.</td>
<td>Equus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEV</td>
<td>Equine encephalosis virus</td>
</tr>
<tr>
<td>EHDV</td>
<td>Epizootic haemorrhagic disease virus</td>
</tr>
<tr>
<td>EIP</td>
<td>Extrinsic incubation period</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
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<td>G</td>
<td>Glycine</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>Hel</td>
<td>Helicase</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IAH</td>
<td>Institute for Animal Health</td>
</tr>
<tr>
<td>IR</td>
<td>Infection rate</td>
</tr>
<tr>
<td>ISVP</td>
<td>Infectious subviral particle</td>
</tr>
<tr>
<td>iu</td>
<td>International unit(s)</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>log_10</td>
<td>Logarithm to the base 10</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>MB</td>
<td>Mouse brain</td>
</tr>
<tr>
<td>MEB</td>
<td>Mesenteronal escape barrier</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>MIB</td>
<td>Mesenteronal infection barrier</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>n.d.</td>
<td>Not done</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>NVC</td>
<td>Non vector-competent</td>
</tr>
<tr>
<td>O'poort</td>
<td>Onderstepoort</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OIE</td>
<td>Office International des Epizooties</td>
</tr>
<tr>
<td>OVI</td>
<td>Onderstepoort Veterinary Institute</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly (A) binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pi</td>
<td>Post infection</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SGEB</td>
<td>Salivary gland escape barrier</td>
</tr>
<tr>
<td>SGIB</td>
<td>Salivary gland infection barrier</td>
</tr>
<tr>
<td>SNT</td>
<td>Serum neutralisation test</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>ST</td>
<td>Serotype</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infective dose</td>
</tr>
<tr>
<td>TOTB</td>
<td>Transovarial transmission barrier</td>
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1 Introduction

1.1 African horse sickness - the disease

African horse sickness (AHS) is an infectious, non-contagious disease of equids. It is caused by African horse sickness virus (AHSV), a ten-segmented, double-stranded RNA virus of the genus Orbivirus, family Reoviridae. Nine serotypes of AHSV have been defined to date. Equids are the principal vertebrate host of the virus, which is transmitted solely by the bite of its blood-feeding arthropod vectors (Wetzel et al., 1970; Meiswinkel, 1998). The lack of horizontal and vertical transmission in the mammalian hosts restricts the disease to areas where suitable vector species are present and to seasons which allow vector activity (Mellor, 1993). AHS was first described after the introduction of domestic horses into sub-Saharan Africa, probably as early as 1569 in East Africa (Barnard, 1998). It causes seasonal epidemics of high morbidity and mortality in susceptible horses.

1.1.1 Geographical distribution and at-risk areas

Endemic areas and recent epidemics of African horse sickness

AHSV is endemic in sub-Saharan Africa and the Sahara desert is thought to be an effective barrier, preventing it establishing itself outside this region (Mellor, 1994). All nine AHSV serotypes are present in the southern part of the continent, where AHSV-9 is considered to be less virulent than the other eight serotypes (Coetzer & Erasmus, 1994; Lord et al., 1998). However, only AHSV-9 has repeatedly been isolated in western Africa and has caused severe outbreaks in this region (Parker et al., 1977). Until the late 1980s it was also the only serotype to be repeatedly identified outside its traditional territory (Mellor et al., 1990b), causing outbreaks on the Arabian Peninsula (1959/60, 1989), the Middle East and India (1959/60) and North Africa (1965/66) (Rafyi, 1961; Diaz Montilla & Panos Marti, 1968; Coetzer & Erasmus, 1994). In 1966, the North African outbreak spread into Spain but it was eradicated in the same year (Diaz Montilla & Panos Marti, 1968). In 1987, another outbreak occurred in Spain, apparently following the importation of infected zebra from Namibia (Lubroth, 1988). The disease, caused by AHSV-4, quickly spread into Morocco and Portugal and
recurred every autumn until it was finally eradicated from this region in 1991 (Mellor et al., 1990a; Rodriguez et al., 1992).

Figure 1-1: Distribution of C. sonorensis and related Culicoides species in North America.
(Wirth & Morris, 1984)

Areas at risk of AHSV

*Culicoides imicola*, an Afro-Asian crepuscular and nocturnal, haematophagous biting midge, is the main field vector of AHSV and is present in all countries where outbreaks have occurred. Its vector competence for orbiviruses in general is underlined by its presence and the transmission of the closely related bluetongue virus (BTV) in an area reaching from Cape Verde in the west to Laos in the east (Mellor & Boorman, 1995). BTV is endemic in most parts of the world between latitudes 35°S and 40°N, although in many areas, other vector species are involved (Mellor & Boorman, 1995). All these areas, including parts of the Americas, Southeast Asia and North Australia, must therefore be considered at risk of AHSV becoming endemic should the virus be introduced (Mellor & Boorman, 1995). On the North American continent, for example, the transmission of BTV by *C. sonorensis* (see Arthropod Vectors, below) indicates that large parts of the continent could also be at risk of AHS should AHSV be introduced.
This species has been shown to be a highly efficient laboratory vector of AHSV (Boorman et al., 1975; Wellby et al., 1996). Figure 1-1 (page 13) shows the distribution of *C. sonorensis* and related *Culicoides* species in North America.

In the northern hemisphere, the distribution of *C. imicola* stretches further north than the regions where BTV is endemic. The presence of the vector means that these regions should also be considered at risk of AHSV. Figure 1-2 shows where the insect has been found in Europe.

![Figure 1-2: Distribution of *C. imicola* in Europe](image)

Provided by Dr. E. Wittmann (2001)

In many areas, outbreaks of AHS are curtailed by the onset of winter. However, AHSV can establish itself for longer periods in zones with a climate that is warm enough to allow overwintering of adult insects. It could also persist in a colder climate if the cold periods are shorter than the maximum viraemic periods of the local vertebrate hosts, i.e. 40 days in the case of zebra, 18 days in the case of horses (Mellor & Boorman, 1995). In contrast, the recent AHS outbreak in the Iberian peninsula showed “silent” periods of 8-12 months, in which time a very low level of vector activity was recorded (Mellor, 1994). Similarly, the disease-free intervals of recent bluetongue (BT) outbreaks in Turkey (1977-1981) and Corsica/Sardinia (2000-2001) lasted for 8-9 months (Yonguc *et al.*, 1982, C. Hamblin, personal communication). These silent
periods considerably exceed the maximum reported viraemic periods of 50 days in sheep and 100 days in cattle (Sellers & Taylor, 1980; MacLachlan et al., 1991; Koumbati et al., 1998) and it is unclear how the virus survived the disease-free periods. However, an alternative overwintering mechanism has been proposed (H. Takamatsu et al., manuscript in preparation): Ovine γδ T-cells have been shown to become persistently infected by BTV. These cells are actively involved in the inflammatory response and therefore migrate to insect biting sites in the skin. It has been shown that contact with skin fibroblasts can transform the persistent infection of γδ T-cells to a lytic infection. Such an infection could result in further replication and release of BTV in the skin areas that are exposed to insect bites and could lead to the infection of Culicoides vectors long after detectable viraemia itself has ceased.

The risk of AHSV establishing itself in Europe was dramatically emphasised by the annual recurrence of the disease in Spain for four years (1987-1990) before it was finally eradicated through a vigorous and costly campaign of vaccination and slaughter (Rodriguez et al., 1992). The recurring and (for some regions) unprecedented BT outbreaks in southern Europe 1998 onwards (Greece, Bulgaria, Sicily, Sardinia, mainland Italy, Corsica and Balearic Isles) have similar implications. C. imicola has been found in all BT-affected regions of Europe except Bulgaria, where C. obsoletus is the dominant species (Dr. P. Mellor, personal communication). It has been suggested that due to climate change, the range of C. imicola in Europe has been extending northward (Rawlings & Mellor, 1994; Rawlings et al., 1998), taking the risk of AHSV and BTV infection with it. Northward spread of C. imicola carries the added danger of exposing other Culicoides species, such as the palaearctic C. obsoletus (see section 1.1.2 below) to the virus, which, if vector-competent, could spread the disease over much of central and northern Europe. Furthermore, a much warmer European climate, as has been predicted by some models, could create novel vector species through the mechanism detailed in section 1.3.4 (page 37).

1.1.2 Hosts and vectors

Mammalian hosts

In southern and eastern Africa, the main if not the only mammalian reservoir hosts of AHSV are zebra species: Equus burchelli and E. zebra in the southern parts, E.
*grevyi* and *E. burchelli* in the eastern parts of the continent (Barnard, 1998). This is considered to be so because in these areas AHSV only seems to be continually present where there are large populations of zebra. Zebras, donkeys, mules and horses can be viraemic and therefore are likely to be infective to blood-feeding arthropods for variable periods after infection, up to 40 days in zebras (Barnard *et al.*, 1994; Mellor & Boorman, 1995; Hamblin *et al.*, 1998). Furthermore, vaccinated horses may be resistant to disease but not necessarily to infection and might therefore become silent carriers of the virus rather like zebra and other non-horse equids (Rodriguez *et al.*, 1992). The epidemiology of the disease appears to be different in western Africa since zebra are absent from this region. However, the virus is still endemic in many parts and the question of the mammalian reservoir is unresolved. It is also interesting to note that of the nine serotypes of AHSV only serotype 9 seems to be able to survive (in west Africa) in the absence of zebra populations. Likely alternative reservoir hosts include the African donkey, the European donkey and mules (Hamblin *et al.*, 1998).

Dogs have been shown to become infected and die after the ingestion of AHSV-infected horseflesh (van Rensburg *et al.*, 1981). However, they are considered to be a dead-end host and of little epidemiological significance since *Culicoides* do not readily feed on them and most authorities consider the induced viraemia to be low level and transient (Braverman & Chizov-Ginzburg, 1996).

Camels, elephants and several ruminant species (cattle, goats, sheep and buffalo) have been reported to produce antibodies against AHSV, but it is not clear whether this seroconversion is due to virus replication or to continuous exposure to antigen because of repeated blood-feeding of AHSV-infected arthropods (Awad *et al.*, 1981a; Barnard *et al.*, 1995). Likewise, it is unclear whether seroconversion in a range of African carnivores (lions, cheetahs, jackals, hyenas and the African wild dog: *Lycaon pictus*) is associated with virus replication (Alexander *et al.*, 1995). There is little evidence that any of these species are of epidemiological significance, although it has been suggested that the camel could successfully replicate the virus (Awad *et al.*, 1981b). The virus has also been isolated from the camel tick (see *Arthropod vectors*, below), which suggests that a separate camel-tick cycle might exist and could have caused the repeated spread of AHSV-9 across the Sahara desert (see section 1.1.1, page 12). It also begs the question whether serotype 9 is better adapted to camels and/or ticks than the other serotypes.
**Arthropod vectors**

Six species of *Culicoides*, three mosquito species and two tick species have been associated with AHSV (Mellor & Boorman, 1995; Wittmann, 2000):

- *C. imicola* and its sibling species *C. bolitinos* have been implicated in outbreaks of AHSV (Mellor, 1990; Baylis et al., 1997; Meiswinkel, 1998; Meiswinkel & Paweska, 1999). Both can be orally infected in the laboratory (Venter et al., 2000), see also chapter 5 (page 107). There is overwhelming evidence that *C. imicola* is the main field vector of AHSV (Mellor & Boorman, 1995). For a more detailed discussion of the role of these insect species in AHSV transmission see section 1.3 (page 35). Figure 1-2 shows the distribution of *C. imicola* in Europe (page 14).

- AHSV was isolated from mixed pools of non-engorged *C. obsoletus* and *C. pulicaris* during the recent outbreak in Spain (Mellor et al., 1990a). *C. obsoletus* has also been associated with BTV (Mellor & Pitzolis, 1979).

- *C. sonorensis* (= *C. variipennis sonorensis*) is the North American field vector of BTV (Foster et al., 1968; Jones et al., 1981) and has been shown to replicate and transmit AHSV following oral infection in the laboratory (Boorman et al., 1975; Wellby et al., 1996).

- *C. nubeculosus* is traditionally considered to be a non-vector species for AHSV and BTV but an increase in larval rearing temperatures has been found to enhance its vector competence for these viruses dramatically (Wellby et al., 1996; Wittmann, 2000; see section 1.3.4, page 37).

- The mosquito species *Anopheles stephensi*, *Culex pipiens* and *Aedes aegypti* have all been shown to transmit AHSV in the laboratory but have never been linked to an outbreak of the disease (Ozawa & Nakata, 1965; Ozawa et al., 1966).

- The camel tick *Hyalomma dromedarii* and the brown dog tick *Rhipicephalus sanguineus sanguineus* have both been shown to transmit AHSV to susceptible horses (Awad et al., 1981b; Dardiri & Salama, 1988). Transstadial transmission from larvae to nymphs and from nymphs to adults was shown for both species, but no transovarial transmission was recorded. The epidemiological significance of ticks remains unclear.
1.1.3 Pathogenesis and clinical signs

Virus is released into the bloodstream of the mammalian host during blood-feeding by an infected arthropod vector. Susceptible horses fall ill after a typical incubation period of two to nine days (Coetzer & Erasmus, 1994). One of the main target tissues of the virus is the endothelium of the host’s blood vessels, which may be severely damaged resulting in excessive loss of vascular fluid into perivascular spaces (Coetzer & Erasmus, 1994). Other tissues can be affected to various degrees, and four clinical patterns have been recognised (Coetzer & Erasmus, 1994). All of them usually are accompanied by a high fever of 40-41°C:

1. Pulmonary AHS (peracute and acute form), featuring rapid development of pulmonary oedema, accompanied by exudation of large amounts of frothy discharge from the nostrils. Death can occur just hours after onset of clinical signs. This form of AHS is common in fully susceptible horses and in dogs. The mortality rate can exceed 90%.

2. Cardiac AHS (subacute form), characterised by severe endocarditis leading to oedema starting at the temporal fossae, eyelids and lips, and later involving the whole head and neck region. The mortality rate can approach 50%.

3. An intermediate, usually acute form, with signs of both pulmonary and cardiac AHS and a mortality rate up to 80%.

4. Horse sickness fever, often clinically inapparent, affecting partially immune horses, but also mules, donkeys and zebra. It also occurs as a reaction to vaccination of horses. There is no mortality.

1.1.4 Control

In endemic areas, control of AHS is achieved by vaccination. In areas where all nine serotypes circulate (e.g. South Africa), live polyvalent attenuated vaccines are used. The most widely used vaccines are two quadrivalent vaccines, containing attenuated serotypes 1, 3, 4, 5 and 2, 6, 7, 8, respectively. Type 9 vaccine is not used since type 6 is cross-protective. Since active immunisation does not afford 100% protection against infection or disease, stabling from before dusk until after dawn is also
practised, often in combination with insect-proofing of stables and use of insect repellents.

In areas where AHSV is not endemic, the emphasis is on maintaining or regaining the disease-free status. In the event of an outbreak, the measures that were taken in the Spanish 1987-1990 outbreak (Rodriguez et al., 1992) are likely to be followed and would therefore start with an immediate ban on the movement of all equids. Stabling and insect control measures can be combined with the use of ring-vaccination and the slaughter or isolation of diseased animals. Ideally, the serotype responsible should be identified as quickly as possible and an appropriate monovalent vaccine produced and used. Traditionally, live poly- and monovalent vaccines have been in use. However, due to the possibility of these viruses replicating and being spread by insect vectors (see also chapter 5, page 107) and the added danger of vaccine viruses reverting to virulence and/or reassorting in the case of polyvalent vaccines, there has been a recent drive towards the use of non-replicative vaccines. Part of this work is to assess the risk of attenuated AHSV strains being transmitted by field vectors.

1.2 African horse sickness virus

1.2.1 Classification

The family Reoviridae

African horse sickness virus is one of 20 virus species within the genus Orbivirus, family Reoviridae (Table 1-1). The nine confirmed genera in this family comprise at least 73 non-enveloped species, including five that have not been assigned yet (Mertens et al., 2000; Dr. P. Mertens, personal communication). The unifying features of the family are a similar structure of the genome, consisting of 10-12 dsRNA segments, and a common replication strategy (Joklik, 1983; Calisher & Mertens, 1998). All family members possess an icosahedral capsid of 70-85 nm diameter that is composed of up to three protein layers. The primary determinant of virus species within the family is an ability to exchange genetic material, i.e. genome segments, during coinfection of the cell. This process, known as reassortment, has been found to correlate well with the more traditional, serology-based methods of species determination and genomic analysis (Mertens et al., 2000). As more information becomes available on less
extensively studied family members, proof of reassortment may supplement other methods of species definition.

Table 1-1: Genera in the family Reoviridae

(Calisher & Mertens, 1998; updated with data from Dr. P. Mertens, personal communication)

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. of genome segments</th>
<th>No. of species (serotypes)</th>
<th>Unassigned viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoreovirus</td>
<td>10</td>
<td>4 (7)</td>
<td>3</td>
</tr>
<tr>
<td>Orbivirus</td>
<td>10</td>
<td>20 (154)</td>
<td>11</td>
</tr>
<tr>
<td>Cypovirus</td>
<td>10</td>
<td>14 (14)</td>
<td>2 and &gt; 160 uncharacterised</td>
</tr>
<tr>
<td>Aquareovirus</td>
<td>11</td>
<td>6 (22)</td>
<td>5</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>11</td>
<td>5 (5)</td>
<td></td>
</tr>
<tr>
<td>Coltivirus*</td>
<td>12</td>
<td>2 (5)</td>
<td>4</td>
</tr>
<tr>
<td>Seadornovirus (proposed)*</td>
<td>12</td>
<td>2 (5)</td>
<td>4</td>
</tr>
<tr>
<td>Fijivirus</td>
<td>10</td>
<td>5 (8)</td>
<td></td>
</tr>
<tr>
<td>Phytoreovirus</td>
<td>12</td>
<td>3 (6)</td>
<td>1</td>
</tr>
<tr>
<td>Oryzavirus</td>
<td>10</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>Entomoreovirus (proposed)</td>
<td>10</td>
<td>5 (8)</td>
<td></td>
</tr>
<tr>
<td>Unassigned viruses</td>
<td>11 or 12</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>110</strong></td>
<td><strong>73 (236)</strong></td>
<td><strong>28</strong></td>
</tr>
</tbody>
</table>

* Based on sequence comparisons, it has been proposed that the genus Coltivirus should be split into two genera, Coltivirus and Seadornavirus (a new genus).

The virus core contains one copy of each of the genome segments. RNA segments have a type one cap structure at the 5' terminus of the positive RNA strand and are phosphorylated at the 5' terminus of the negative strand, but they are not polyadenylated at the 3' end (Chow & Shatkin, 1975; Shatkin & Kozak, 1983). The virus core also contains the virus-encoded RNA polymerase, a helicase and capping and methylation enzymes (Joklik, 1983).

The genus Orbivirus

The genus Orbivirus was originally proposed to accommodate a group of arboviruses which shared several characteristics (Borden et al., 1971; Murphy et al., 1983).
One of these characteristics, high resistance to detergents, suggested the absence of a lipoprotein envelope, setting this group apart from other arboviruses (Borden et al., 1971). Although orbiviruses do share a number of features with orthoreoviruses, such as resistance to organic solvents and a ten-segmented dsRNA genome (Verwoerd, 1969; Verwoerd, 1970), they were also found to differ in their ecology (obligate arthropod transmission) and their acid lability (Owen, 1964; Stanley, 1967). The creation of a new genus for the orbiviruses was further justified by the discovery that they possess an icosahedral core particle with a T=13 (1) surface arrangement (Hewat et al., 1992).

Rotaviruses possess a very similar inner capsid layer but they are not arthropod-borne viruses.

Table 1-2: Virus species in the genus Orbivirus
(using data from Mertens et al., 2000)

<table>
<thead>
<tr>
<th>Virus Serogroup (Species)</th>
<th>Serotypes</th>
<th>Host Species</th>
<th>Principal vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-African horse sickness virus species (AHSV)</td>
<td>AHSV-1 to 9</td>
<td>Equids, dogs, elephants, camels, cattle, sheep, goats, predatory carnivores and in special circumstances humans</td>
<td>Culicoides</td>
</tr>
<tr>
<td>2-bluetongue virus species (BTV)</td>
<td>BTV-1 to 24</td>
<td>Cattle, sheep, goats, camels. Elephants, (domestic and wild ruminants), predatory carnivores.</td>
<td>Culicoides</td>
</tr>
<tr>
<td>3-Changuinola virus species (CGLV)</td>
<td>Twelve &quot;named&quot; serotypes</td>
<td>Humans, rodents, sloths.</td>
<td>Phlebotomine flies, mosquitoes</td>
</tr>
<tr>
<td>4-Chenuda virus species (CNUV)</td>
<td>Seven &quot;named&quot; serotypes</td>
<td>Seabirds</td>
<td>Ticks: Ornithodoros; Argas</td>
</tr>
<tr>
<td>5-Chobar Gorge virus species (CGV)</td>
<td>Two &quot;named&quot; serotypes</td>
<td>Bats</td>
<td>Ticks: Ornithodoros</td>
</tr>
<tr>
<td>6-Corriparta virus species (CORV)</td>
<td>Three &quot;named&quot; serotypes</td>
<td>Humans, rodents.</td>
<td>Culicine mosquitoes</td>
</tr>
<tr>
<td>7-Epizootic haemorrhagic disease virus species (EHDV)</td>
<td>EHDV-1 to 8</td>
<td>Cattle, sheep, deer, camels, llamas, wild ruminants, marsupials.</td>
<td>Culicoides</td>
</tr>
<tr>
<td>8-Equine encephalosis virus species (EEV)</td>
<td>EEV 1 to 7</td>
<td>Equids</td>
<td>Culicoides</td>
</tr>
<tr>
<td>9-Eubenangee virus species (EUBV)</td>
<td>Four &quot;named&quot; serotypes</td>
<td>Unknown (isolated from insect vectors)</td>
<td>Culicoides and Anopheline, Culicine mosquitoes.</td>
</tr>
<tr>
<td>10-Ieri virus species (IERIV)</td>
<td>Three &quot;named&quot; serotypes</td>
<td>Birds</td>
<td>Culex mosquitoes</td>
</tr>
<tr>
<td>11-Great Island virus species (GIV)</td>
<td>Thirty six &quot;named&quot; serotypes</td>
<td>Seabirds, rodents, humans.</td>
<td>Ticks: Argas, Ornithodoros, Isobodes.</td>
</tr>
<tr>
<td>Virus Serogroup (Species)</td>
<td>Serotypes</td>
<td>Host Species</td>
<td>Principal vector</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------</td>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>13-Orungo virus species (ORUV)</td>
<td>ORUV-1 to 4</td>
<td>Humans, camels, cattle, goats, sheep, monkeys</td>
<td>Culicine mosquitoes.</td>
</tr>
<tr>
<td>14-Palyam virus species (PALV)</td>
<td>Eleven &quot;named&quot; serotypes</td>
<td>Cattle, sheep</td>
<td>Culicoides and Culicine mosquitoes</td>
</tr>
<tr>
<td>15-St Croix River Virus (SCRV)</td>
<td>SCRV-1</td>
<td>unknown hosts</td>
<td>ticks</td>
</tr>
<tr>
<td>16-Umatilla virus species (UMAV)</td>
<td>Four &quot;named&quot; serotypes</td>
<td>Birds</td>
<td>Culicine mosquitoes</td>
</tr>
<tr>
<td>17-Wad Medani virus species (WMV)</td>
<td>Two &quot;named&quot; serotypes</td>
<td>Domestic animals</td>
<td>Ticks: Boophilus, Rhipicephalus, Hyalomma, Argas.</td>
</tr>
<tr>
<td>18-Wallal virus species (WALV)</td>
<td>Two &quot;named&quot; serotypes</td>
<td>Marsupials</td>
<td>Culicoides.</td>
</tr>
<tr>
<td>19-Warrego virus species (WARV)</td>
<td>Two &quot;named&quot; serotypes</td>
<td>Marsupials</td>
<td>Culicoides and Anopheline, Culicine mosquitoes</td>
</tr>
<tr>
<td>20-Wongorr virus species (WGRV)</td>
<td>Eight &quot;named&quot; or &quot;numbered&quot; serotypes</td>
<td>Cattle, macropods</td>
<td>Culicoides and mosquitoes</td>
</tr>
</tbody>
</table>

Tentative species and unassigned viruses within the genus

| Andasibe virus (ANDV) | unknown hosts | mosquitoes |
| Ife virus (IFEV) | rodents, birds, ruminants | mosquitoes |
| Itupiranga virus (ITUV) | unknown hosts | mosquitoes |
| Japanaut virus (JAPV) | unknown hosts | mosquitoes |
| Kammavanpettai virus (KMPV) | birds | unknown |
| Lake Clarendon virus (LCV) | birds | ticks |
| Matucare virus (MATV) | unknown hosts | ticks |
| Tembe virus (TMEV) | unknown hosts | mosquitoes |
| Codajas virus | rodents | mosquitoes |
| Tracambe Virus | unknown hosts | mosquitoes |
| Peruvian horse virus | Culicoides | horses |
| Peruvian Rodent virus (PC21) | unknown hosts | rodents |

Total 162

1- The 36 virus isolates, currently grouped within the Great Island virus species, as well as the 8 virus isolates in the Wongorr virus species have not been extensively compared in serum neutralisation assays. However, preliminary serological studies of the GIV species indicate that they may represent different serotypes.

2- Chobar Gorge and Fomede are related but distinct as analysed by complement fixation tests. These viruses form the Chobar Gorge virus serogroup (species).

3- leri, Arkonam and Gomoka are closely related viruses and form the leri virus serogroup (species).

4- The newly recognised Great Island virus species contains virus isolates previously classified in the Kemerovo virus and Great Island virus complexes of the Kemerovo serogroup.

*= The precise assignment of the serotype numbers to the previously "named" serotypes was proposed by Howell et al. (2002).
1.2.2 Virus structure

All orbiviruses contain 10 segments of dsRNA, surrounded by two capsid shells. The inner capsid, made up from the inward-facing virus protein VP3(T2) and the outward-facing VP7(T13), encloses the 10 segments of RNA and the minor proteins VP1(Pol), VP4(Cap) and VP6(Hel), which are the core associated enzymes. The virus structure of the prototype *Orbivirus* species BTV is illustrated in Figure 1-3 (page 24) and the genome assignment and orbivirus protein functions are summarised in Table 1-3 (page 25) and described in section 1.2.4 (page 30). VP7 is the main species (i.e. serogroup) specific antigen, common to all AHSV serotypes. Similarly, VP3 is highly conserved across the serogroup, but neither of these proteins is serologically cross-reactive with the corresponding proteins of other *Orbivirus* species. The outer capsid layer consists of VP2 and VP5. VP2 is involved in cell entry and in combination the two proteins determine the serotype (defined by serum neutralisation test-SNT) of a given virus (Roy *et al.*, 1994). By analogy to BTV, VP2 is thought to interact directly with neutralising antibodies while VP5 exerts an indirect effect on neutralisation by modulating VP2 conformation (Cowley & Gorman, 1989; Mertens *et al.*, 1989). More recently, AHSV and BTV VP5 have both been shown to directly interact with neutralising antibodies, although on a lesser scale than VP2 (Martinez-Torrecuadrada *et al.*, 1999; DeMaula *et al.*, 2000). VP5 must therefore be considered a co-determinant of serotype.
The virus RNA genome segments are numbered one to ten according to their migration order in agarose gel electrophoresis (Table 1-3, page 25). Apart from the seven structural proteins VP1-VP7 there are three virus-encoded proteins that cannot be routinely isolated from mature virus particles and are therefore termed “nonstructural proteins” (NS1 to NS3). Segments five, nine and ten each code for more than one protein. These proteins are symbolised by VP5/5a, VP6/6a/6b/6c and NS3/3a, respectively (Roy et al., 1994).
Table 1-3: Proteins of bluetongue virus and related orbiviruses

(available from http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/Orbivirus.htm)

<table>
<thead>
<tr>
<th>Genome segment (Size: bp)</th>
<th>Protein nomenclature (§: protein structure/function)</th>
<th>Location</th>
<th>Copy number/particle</th>
<th>Number of amino acids</th>
<th>Dalton</th>
<th>Properties and Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3954)</td>
<td>VP1 (Pol)</td>
<td>Within the sub-core at the 5 fold axis</td>
<td>11*</td>
<td>1302</td>
<td>14958</td>
<td>RNA dependent RNA polymerase (§: Pol)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trimer, controls virus serotype, serotype specific antigen, cell attachment protein, outer layer of the outer capsid, contains neutralising epitopes, most variable protein, involved in determination of virulence, cleaved by proteases.</td>
</tr>
<tr>
<td>2 (2926)</td>
<td>VP2</td>
<td>Outer capsid</td>
<td>176*</td>
<td>956</td>
<td>111112</td>
<td>Innermost protein capsid shell, sub-core capsid layer, T=2 symmetry (§: T2), self assembles, retains icosahedral symmetry by itself, controls size and organisation of capsid structure, RNA binding, interacts with internal minor proteins. Highly conserved protein. Physical organisation of genome.</td>
</tr>
<tr>
<td>3 (2770)</td>
<td>VP3 (T2)</td>
<td>Sub-core capsid layer (T=2 symmetry)</td>
<td>120</td>
<td>901</td>
<td>103344</td>
<td>Dimers, capping enzyme (§: Cap), guanylyl transferase, transmethylase 1 and 2.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Forms tubules of unknown function in the cell cytoplasm. These tubules are a characteristic of orbivirus replication.</td>
</tr>
<tr>
<td>4 (2011)</td>
<td>VP4 (Cap)</td>
<td>Within the sub-core at the 5 fold axis</td>
<td>9*</td>
<td>654</td>
<td>76433</td>
<td>Inner layer of the outer capsid, glycosylated, helps control virus serotype, variable protein, trimer.</td>
</tr>
<tr>
<td>5 (1769)</td>
<td>NS1 (TuP)</td>
<td>Cytoplasm, Forms tubules (abundant in cell cytoplasm)</td>
<td>0</td>
<td>552</td>
<td>64445</td>
<td>Trimer, forms outer core surface, T=13 symmetry (§: T13), in some species (AHSV) it can form flat hexagonal crystals made up of layers of hexameric rings of trimers, involved in cell entry, involved in high core infectivity in vector insect and cells, reacts with “core neutralising” antibodies. Immuno-dominant major serogroup specific antigen.</td>
</tr>
<tr>
<td>6 (1638)</td>
<td>VP5</td>
<td>Outer capsid</td>
<td>360</td>
<td>526</td>
<td>59163</td>
<td>Important viral inclusion body matrix protein, ssRNA binding, phosphorylated. Can be associated with outer capsid.</td>
</tr>
<tr>
<td>7 (1156)</td>
<td>VP7 (T13)</td>
<td>Outer core (T=13 symmetry)</td>
<td>280</td>
<td>349</td>
<td>38458</td>
<td>Glycoproteins, membrane proteins, involved in cell exit, in some genera (AHSV) these ar variable proteins and may be involved in determination of virulence. Cytotoxic, can disrupt cell membranes.</td>
</tr>
<tr>
<td>8 (1124)</td>
<td>NS2 (ViP)</td>
<td>Cytoplasm, viral inclusion bodies (VIB) (can be associated with outer capsid)</td>
<td>0</td>
<td>357</td>
<td>40999</td>
<td>ssRNA and dsRNA binding, helicase, NTPase.</td>
</tr>
<tr>
<td>9 (1046)</td>
<td>VP6 (Hel)</td>
<td>Within the sub-core at the 5 fold axis</td>
<td>72</td>
<td>328</td>
<td>35750</td>
<td>Trimer, controls virus serotype, serotype specific antigen, cell attachment protein, outer layer of the outer capsid, contains neutralising epitopes, most variable protein, involved in determination of virulence, cleaved by proteases.</td>
</tr>
<tr>
<td>10 (822)</td>
<td>NS3 NS3a</td>
<td>Cell Membranes (not abundant in mammalian cells)</td>
<td>229</td>
<td>216</td>
<td>25572</td>
<td>Important viral inclusion body matrix protein, ssRNA binding, phosphorylated. Can be associated with outer capsid.</td>
</tr>
</tbody>
</table>

*Based on ratios of the structural proteins as estimated (Huismans and Van Dijk, 1990), which have been normalised to 780 copies of VP7 (T13) per particle. Protein numbers per particle that have been confirmed by analysis of particle atomic structure are shown in bold underlined. §Based on the genome segment order during agarose gel electrophoresis, as determined by Pedley et al. (1988), the RNA segment nomenclature proposed by Gorman et al. (1985) and the gene coding assignment by Mertens et al. (1984). §: Protein structure/function, to facilitate identification and comparisons of proteins across species, genera and families of dsRNA viruses, as suggested by Grimes et al. (1998) and Gouet et al. (1999). Pol= RNA polymerase; Cap= capping enzyme (guanylyltransferase); Hel= helicase enzyme; T2= protein with T=2 symmetry; T13= Protein with T=13 symmetry; ViP= Viral Inclusion body matrix protein; TuP= tubule protein; AHsV= African horse sickness virus.
1.2.3 Nonstructural protein NS3/3a

Structure and function

The two nonstructural proteins NS3 and NS3a are encoded from two in-phase overlapping reading frames of segment 10 and vary only in 10 additional amino acids at the amino terminus of NS3 (van Staden & Huismans, 1991). In BTV, both proteins seem to be synthesised in equimolar amounts and nothing is known about possible functional differences between them. They are therefore collectively referred to as NS3/3a in this thesis. NS3/3a is a transmembrane protein with two highly conserved hydrophobic regions. Experiments with AHSV have shown that mutations inserted into these regions, turning them hydrophilic and thus unable to “bridge” the cell membrane, reduce their cytotoxic effect on insect cells (van Staden et al., 1995; van Staden et al., 1998). In BTV, the protein contains two potential glycosylation sites, one of them between the two transmembrane regions (Roy, 1992; Roy et al., 1994).

Studies using recombinant baculovirus expression systems show that BTV-like particles can be synthesised but are not released from the cells in the absence of NS3/3a. Addition of a baculovirus expressing NS3/3a results in the successful release of virus like particles from these cells (Hyatt et al., 1993). This experiment strongly suggests that the presence of NS3/3a is vital for the escape of virions from the insect cell. It may perform a similar function in mammalian cells. A role of NS3/3a in virus release is also indicated by the discovery of two potential ubiquitin-ligase recruiting motifs in BTV NS3/3a (Strack et al., 2000). The two motifs, PPRY at NS3 amino acid (aa) 36-39 and PSAP at aa 41-44, are identical with retroviral “L (=late assembly) domains”. The late assembly domains PPxY (oncoretroviruses, e.g. Rous sarcoma virus) and P(T/S)AP (lentiviruses, e.g. Simian immunodeficiency virus) are located on the Gag polyprotein and have been shown to recruit ubiquitin ligase activity, resulting in Gag-ubiquitination (Strack et al., 2000). The insertion of point mutations into the motifs abolished ubiquitination and prevented budding of retrovirus-like-particles from the cell. Proteasome-inhibition with lactacystin also inhibited budding, presumably via ubiquitin-depletion of the cell (Strack et al., 2000). It is concluded by the authors that L domains are essential for virus exit in retroviruses and fulfil their role by way of protein-ubiquitination, possibly involving cell membrane proteins that are normally involved in endocytosis by mono-ubiquitination. While retroviruses were shown to
possess either one of the two motifs, BTV, Broadhaven virus and Palyam virus NS3/3a were shown to contain both motifs in very close proximity, overlapping in the case of Broadhaven virus (Table 1-4). A very similar motif in Ebola virus was shown to be particularly effective when the sequence was inserted into chimaeric retrovirus like particles (VLPs).

Table 1-4: Candidate L domains in some orbivirus NS3
(Strack et al., 2000)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
<th>Position, amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broadhaven virus</td>
<td>SPITA PAYA</td>
<td>10-18</td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>TISQPPRYA SALAM</td>
<td>32-45</td>
</tr>
<tr>
<td>Palyam virus</td>
<td>VPYQPPAY PTVY</td>
<td>21-33</td>
</tr>
</tbody>
</table>

It has been suggested that variation in NS3/3a may affect the timing and titre of AHSV release from insect cell culture (Martin et al., 1998). Exchange of this protein has been shown to modify virulence in a mouse model system. For the latter studies, reassortant viruses were used (O'Hara et al., 1998).

Involvement of NS3/3a in virus release from cultured insect cells suggests that it might be involved in the mesenteronal escape barrier (see section 1.3.5, page 39). It has been shown for BTV that many individuals from competent insect vector colonies do not develop a disseminated virus infection but harbour an infection localised to the midgut cells, the primary target tissue for orally ingested virus. The mechanism of this “trapping” of virus is not well understood but may relate to the exit mechanism. In that case, there is a real possibility that variation in NS3/3a could affect the existence or non-existence of a mesenteronal escape barrier in a given virus-insect combination, i.e. co-defines vector competence of the insect for a particular virus strain.

NS3/3a sequence variation

One of the most striking features of AHSV is the high level of variability in segment 10 compared to the homologous segment 10 genes in BTV, Palyam virus and epizootic haemorrhagic disease virus (EHDV) (Sailleau et al., 1997; Martin et al., 1998;
van Niekerk et al., 2001b). The degree of variability is illustrated by a rootless phylogenetic tree of the predicted AHSV NS3 protein sequences (Figure 1-4, page 29). On the basis of their sequences, isolates of segment 10 and NS3 can be assigned to one of three distinct groups (Sailleau et al., 1997). Early work suggested a firm relationship between serotype and NS3/3a, with serotypes 4, 5, 6 and 9 clustering into the α group, serotypes 3 and 7 grouping into the β group and serotypes 1, 2 and 8 falling into the γ group (Sailleau et al., 1997). However, orbiviruses can reassort, i.e. exchange segments of RNA during co-infection of cell cultures. Prolonged viraemia in zebra and lifelong infection of susceptible insects enhance the chance of reassortment taking place. Almost 100% of zebra in the Kruger National Park (South Africa) have been shown to seroconvert for at least five serotypes of AHSV in their first year of life (Lord et al., 1998). This makes co-infection with two or more serotypes very likely in at least some animals. Culicoides vectors become persistently infected and may take several blood meals in their lifetime. These are ideal conditions for reassortment to take place. Therefore, the relationship between the type of segment 10 (α, β, γ) and serotype, as determined by genome segments 2 and 6, may be a relatively unstable one. Indeed, recent work shows that isolates of AHSV-8 cluster into various groups of NS3/3a, providing strong evidence that reassortment does take place in the field (van Niekerk et al., 2001b). Analysis of NS3 sequences available on protein databases shows that isolates of serotype 1 also cluster into more than one group (Figure 1-4, page 29). If reassortment does take place to an appreciable extent it must be considered a powerful factor in AHSV evolution. Variants in segment 10 can only be expected to survive in two scenarios. One is that variation represents an adaptation to selective pressures in the past, but these factors have subsequently disappeared and variants now have no selective advantage over each other. They are therefore passed on with constant frequencies. The other scenario is that variation does represent adaptation to present environmental pressures and variants survive because each of them has found an ecological niche. In view of the likely involvement of NS3/3a in cell exit by budding and the absence of lysis or cytopathic effect (CPE) in the Culicoides cell culture, it is thought likely that NS3/3a is essential for virus dissemination and possibly the vector status of the insect. Variations in NS3/3a could therefore reflect adaptation to distinct vector insect species or populations.
Figure 1-4: Variation in AHSV NS3
Unrooted neighbour-joining tree of AHSV NS3 protein sequences. Sequences were obtained from SwissProt and TrEMBL databases. For each isolate, the serotype (ST) is given, followed by the accession number. Bar (bottom left) represents 1% variation. The tree was designed using the "distances" program in GCG 10. Three distinct groups are labelled as alpha, beta, and gamma.
The replication cycle of AHSV is thought to resemble that of BTV and indeed all other members of the family Reoviridae. Properties shared by all these viruses include the possession of dsRNA, the composition of their genome of RNA segments, the lack of complete uncoating and the possession of their own enzymes transcribing dsRNA into mRNA (Zarbl & Millward, 1983). Orbi-and coltiviruses differ from other family members in the formation of characteristic cytoplasmic tubules during replication. These tubules are formed by NS1 (Eaton et al., 1990). The following outline of virus replication is mainly based on the BTV replication cycle and primarily relies on observations made in mammalian cells (Figure 1-5).

Adsorption

Virus binding to an unknown receptor is mediated by VP2 (Huijsmans et al., 1983). It was also shown that BTV particles lacking VP2, but containing VP5, could not bind to BHK cells in suspension (Huijsmans et al., 1983). The importance of VP2 for
successful infection is underlined by the finding in AHSV-4 that immunisation with this protein protects horses against experimental infection (Stone-Marschat et al., 1996). Cleavage of VP2 by trypsin, chymotrypsin or species-specific mammalian serum proteases results in production of infectious subviral particles (ISVPs) with enhanced infectivity for Culicoides midges (Marchi et al., 1995; Mertens et al., 1996). Core particles, which lack VP2 and VP5, have been shown to have only low levels of infectivity for mammalian cells but to have an infectivity similar to whole virus particles for insect cells (Mertens et al., 1996). This suggests that removal of VP2 and VP5, and the exposure of the core surface layer allows VP7 to mediate an alternative attachment process in Culicoides insects. This view is supported by cell attachment experiments (Xu et al., 1997) and observations of BTV core particle neutralisation by anti-VP7 antibodies (Hutchinson, 1999). It has been hypothesised that a mutual adaptation may exist between regional Culicoides populations and VP7 sequences of local BTV strains in endemic areas but no supporting evidence for this has been found (Wilson et al., 2000). However, the high variability of VP2 and VP5 allows for speculation on the potential adaptation of these proteins to Culicoides receptor variation.

Cell entry and uncoating

Up to three independent pathways for the initiation of infection by BTV may exist. Intact virus particles have been observed entering the cell via receptor-mediated endocytosis (Eaton et al., 1990). ISVPs have been associated with shorter eclipse periods, suggesting a non-endosomal route of entry, possibly by direct plasma membrane penetration (Eaton et al., 1990). However, in more recent experiments, both BTV virus particles and ISVPs have been found to require a “low pH step” in order to productively infect mammalian and insect cells, strongly suggesting that they both use the endosomal pathway of infection (Hutchinson, 1999). In contrast, BTV core infection does not require a low endosomal pH, possibly because cores have already lost the acid labile outer capsid proteins (Hutchinson, 1999). Core particles can infect host cells via a VP7-mediated pathway that appears to utilise an alternative cell receptor. Cores have been shown to bind to cell surface glycosaminoglycans (Hutchinson, 1999).

Partial uncoating, i.e. removal of VP2 and probably VP5 from BTV virions, takes place in endosomes (Huismans et al., 1983; Eaton et al., 1990). Core particles are
released from the endosome by an unknown mechanism before endosome fusion with lysosomes takes place. Cores appear in the cell cytoplasm within an hour of infection with virus particles. Removal of both outer capsid proteins is required for the virus transcriptase to be activated (van Dijk & Huismans, 1980).

**Transcription of the parental genome**

Virus dsRNA cannot act as mRNA and host cell polymerases are unable to transcribe from dsRNA. Therefore, orbivirus particles need to supply their own transcription enzymes. They also provide enzymes necessary for capping and methylation of the 5'RNA termini (Table 1-3, page 25).

The virus polymerase becomes active once partial uncoating, i.e. the removing of outer capsid proteins has taken place. As in most members of the *Reoviridae*, complete uncoating of virions does not occur in orbiviruses and the genome remains within the core. BTV dsRNA transcription is fully conservative, i.e. parental strains are not found amongst transcription products. Transcription is asymmetric, i.e. only one parental strain is transcribed and results in full-length ssRNA copies. Each virus particle contains approximately ten copies of polymerase, so it is thought likely that each parental RNA segment is transcribed by only one molecule of the polymerase (Stuart et al., 1998). ssRNAs transcribed from parental cores are capped at the 5' end but not adenylated at the 3' terminus (Zarbl & Millward, 1983; Eaton et al., 1990). They serve two purposes, to act as mRNA for virus protein synthesis and as a template for the synthesis of negative-strand RNA within nascent progeny virus particles.

**Formation of progeny double-stranded RNA**

Capped, positive-strand ssRNA molecules, transcribed from parental cores, are used as a template for one round of negative-strand synthesis. The negative strand remains with its template to re-form the dsRNA genome segments. This process takes place inside nascent core particles, which are precursors of progeny virus particles. ssRNA→dsRNA polymerase activity, like dsRNA→ssRNA activity, is provided by VP1.
Transcription from the progeny double-stranded RNA

Messenger RNA that is transcribed from parental cores is also referred to as "early" mRNA, while the mRNA from progeny cores is "late" mRNA. These two types of mRNA differ in the timing of their synthesis and possibly in their structure. In BTV, early mRNA is mainly found at about two to six hours after infection, while late mRNA is synthesised from approximately four hours post infection and reaches a maximum at around 12 hours post infection (Eaton et al., 1990). In Orthoreovirus progeny cores, guanylyl transferase, transmethylases and capping enzymes are inactive. This corresponds with a switch in cell metabolism from cap dependent to non-cap dependent. Uncapped transcripts do not serve as templates for minus-strand synthesis (Zarbl & Millward, 1983). In rotavirus replication, host cell shut-off occurs as a result of the poly(A) binding protein (PABP) eviction from the eIF4F complex, mediated by the binding of the non-structural protein NSP3 to eIF4GI (Piron et al., 1998). Although it is unknown whether the same mechanisms exist in Orbivirus species, observations of inhibited cap dependent translation late in BTV infection of mammalian cells suggest a mechanism for host cell shut-off (Stirling, 1996). This is observed from approximately 12 h post infection. The absence of a reduction in host protein synthesis in C. sonorensis (KC) cells correlates with a persistent infection, the absence of CPE in these cells and the tolerance of Orbivirus infections by Culicoides midges (Fu, 1996; Stirling, 1996).

Translation of virus messenger RNA

During replication of Orthoreovirus, early mRNA is capped and can therefore compete with host cell mRNA for translation at the ribosome (Zarbl & Millward, 1983). Late virus mRNA is uncapped and this coincides with a virus-induced switch in the translational apparatus from cap dependent to non-cap dependent. As a result host cell mRNA, as well as early virus mRNA, cannot be translated. The consequence is shutdown of host cell protein synthesis and high efficiency of virus mRNA translation, due to absence of competition (Zarbl & Millward, 1983). It is possible that an equivalent mechanism exists in orbiviruses such as BTV and AHSV but the viral proteins involved have not yet been identified.
Morphogenesis

The process of orbivirus assembly is not well understood but starts with the formation of sub-core particles containing ssRNA, which is sensitive to ribonuclease activity (Eaton et al., 1990). The process that selects exactly one copy of each ssRNA segment for packaging into each nascent sub-core particle is not currently understood, but it is at this stage that genome segment reassortment must take place. Replication of genome segments and addition of viral polypeptides to the nascent particles result in the formation of core particles that contain ribonuclease-resistant dsRNA. It is significant for the process of core formation that virus proteins VP1, VP3, VP4, VP6 and VP7 are self-assembling (French & Roy, 1990; Loudon & Roy, 1991). Experiments using baculovirus expression systems show that VP3 and VP7 can form core-like particles in the absence of other virus proteins or viral RNA (French & Roy, 1990; Maree et al., 1998). VP2 is probably added to progeny cores at the periphery of virus inclusion bodies (VIBs, see Virus inclusion bodies and superinfection, below). VP5 is added at the same point or possibly even later (Eaton et al., 1990). If co-expressed with VP3 and VP7, VP2 and VP5 also self-assemble to form virus-like particles (French et al., 1990).

Virus exit from the cell

There are probably three mechanisms for the release of orbiviruses from cells. Budding is associated with the acquisition of a temporary virus envelope and is common in insect cells but virions can also exit through the cell membrane without becoming enveloped (Eaton et al., 1990). Large quantities of virus are also released following cell lysis in mammalian cells. The presence of NS3/3a is necessary for virus exit from insect cells to occur (Hyatt et al., 1993). It has also been shown to modify virulence in a mammalian model, suggesting that it might perform similar functions in equine hosts (O'Hara et al., 1998). The role of NS3/3a is discussed in section 1.2.3 (page 26).

Virus inclusion bodies and superinfection

After leaving the endosome, parental core particles bind to intermediate filaments, start transcription and become associated with a matrix called a “virus inclusion body” (VIB) precursor. The matrix is thought to be formed by condensation of virus proteins translated on ribosomes outside the matrix, with viral ssRNA transcribed
by core particles. Continued aggregation of virus proteins and RNA leads to complete surrounding of the parental core particle by the matrix and a VIB is formed. Within this VIB, progeny core particles assemble. The nonstructural protein NS2, a nonspecific ssRNA-binding, multimer-forming protein, is common in the VIB and thought to be important in the genome replication and assembly of progeny cores (Huismans \textit{et al.}, 1987; Theron & Nel, 1997). The addition of VP2 and VP5 to progeny cores means that the resulting progeny virus particles cannot transcribe, thus slowing down the virus replication in the host cell. In cell culture, BTV has been shown to circumvent this obstacle via superinfection of the cell by progeny virus particles following their release (Hyatt \textit{et al.}, 1989). Consequently, the reinforcing progeny virus particles are stripped of their outer capsid proteins and become transcriptionally active core particles. Since persistently infected insect cells only contain a single large VIB (Dr. P. Mertens, personal communication) it is likely that these particles, or the VIB they produce, fuse with the existing VIB. Such close association may aid the occurrence of genome reassortment.

\textit{Peculiarities of virus replication in insect cells}

No CPE is observed in insect cell culture derived from the BTV vector \textit{C. sonorensis} (KC cells). These cells will survive for at least three weeks following infection with BTV or AHSV. There is also no evidence to suggest that the vector insects themselves are adversely affected by infection with BTV or AHSV. Therefore, host protein synthesis is not shut down in insect cells and cell lysis is not used as a way of virus exit from the cell. A transformation of RNA translation to a non-cap dependent mode appears very unlikely to occur in insect cells.

\subsection*{1.3 Culicoides biting midges and AHSV transmission}

\textit{Culicoides biting midges}

\textit{Culicoides} (Diptera: Ceratopogonidae) are a genus of small (1-3 mm), mainly haematophagous insects (Meiswinkel \textit{et al.}, 1994). They inhabit all major landmasses except for the polar regions and a few geographically isolated island groups, such as New Zealand and the Hawaiian archipelago (Boorman, 1993; Meiswinkel \textit{et al.}, 1994). Approximately 1200 species have been identified but many more are thought to exist.
Life cycle

The complete life cycle of *Culicoides* includes egg, four larval stages, pupa and adult (imago). Depending on species and climate, the cycle takes between seven days and seven months to complete and in temperate regions, the longer periods often include a diapause of the fourth stage instar larva (Kettle, 1984). *Culicoides* are also thought to overwinter as inactive adults in frost-free areas of temperate countries (Rawlings & Mellor, 1994). Immature stages depend on the presence of moisture and organic matter (swamps, damp soil, animal dung etc.) for their development and the absence of suitable breeding grounds can be a limiting factor for a species' distribution and abundance (Meiswinkel et al., 1994). Females of the haematophagous species require a blood meal from a warm-blooded animal for each batch of eggs to mature while the males never blood-feed. Blood-feeding will usually take place once every few days in a lifespan that can last from a few days to over four weeks, dependent on ambient temperature, humidity, wind speeds etc (Baylis et al., 1998).

Economic and public health importance

As a biting nuisance, *Culicoides* can seriously interfere with human outdoor activity (e.g. in parts of Scotland). They also cause sweet itch, a severe allergic dermatitis that can make horse-keeping virtually impossible in affected areas. *Culicoides*-transmitted animal parasites include the economically significant nematode *Onchocerca cervicalis* of horses and a range of nematodes and protozoa of various mammalian and avian species (Linley, 1985).

Although *Culicoides* species also transmit Oropouche virus, which causes a flu-like disease of humans in Latin America (Anderson et al., 1961), their greatest significance is as vectors of animal viruses. Over 50 viruses have been isolated from *Culicoides* insects (Meiswinkel et al., 1994), among them AHSV, BTV, EHDV and equine encephalosis virus (EEV). AHSV and BTV are such a threat to livestock that the diseases these viruses cause have received OIE list A status.

1.3.2 Association of *Culicoides* with AHSV

*Culicoides imicola* was first implicated in the transmission of AHSV by Du Toit in 1944 (Wetzel et al., 1970). The virus has been repeatedly isolated from blood-free *C. imicola* catches in areas where AHS was reported (Mellor et al., 1990a; Baylis et al.,
1997; Meiswinkel, 1998) and this species has been shown to become persistently infected in the laboratory (Venter et al., 2000). Furthermore, the time of C. imicola abundance has been linked with the timing of AHS epidemics (Ortega et al., 1998). This leaves little doubt that C. imicola is the most important vector species of AHSV (Mellor et al., 2000).

More recently, Culicoides bolitinos, a sibling species of C. imicola, has also been implicated in AHS outbreaks (Meiswinkel & Paweska, 1999). C. bolitinos is abundant in areas of southern Africa considered too dry and cool to sustain large populations of C. imicola, and may therefore extend the at-risk area for AHS (Venter & Meiswinkel, 1994; Venter et al., 2000).

1.3.3 Vector capacity and vector competence

Vector capacity describes the ability of an insect vector population to transmit a pathogen to the vertebrate hosts. The main determinants of vector capacity are the size of the vector population, the number of blood meals taken by a vector per host per day, the daily survival rate of the vectors, the length of the extrinsic incubation period and vector competence (Mullens, 1991). The extrinsic incubation period (EIP) is the time span from a vector's consumption of an infectious blood meal to the onset of the vector's transmissiveness during a subsequent blood meal.

Vector competence describes the proportion of individuals in a vector population that are capable of becoming transmissive, if infected, with a given pathogen. Applied to an individual insect, it denotes the ability or otherwise of this insect to become infected with a pathogen and transmit it to a suitable vertebrate host after the EIP. Vector competence depends on vector genotype, environmental factors and the nature of the infecting pathogen. This work investigates how variation in Culicoides vector genotype and variation in AHSV genome can affect Culicoides vector competence, measured as the proportion of insects that become infected (infection rate) or develop a fully disseminated infection (termed "disseminated-infection rate" in this thesis).

1.3.4 Replication of orbiviruses in Culicoides insects

More research has been carried out on BTV than on any other orbivirus, but events in the vector-competent insect are thought to be very similar for all these viruses (Mellor & Boorman, 1995). After oral ingestion of the virus during a blood meal from a
viraemic vertebrate host and deposition of the blood meal in the midgut, virus particles infect the midgut cells from the luminal side (Figure 1-6, page 40). Cleavage of VP2 by mammalian serum proteases or insect gut proteases may result in production of ISVPs with increased infectivity for Culicoides midges (Marchi et al., 1995; Mertens et al., 1996). The virus replicates within the midgut cells and progeny virus particles are released through the basement membrane on the abluminal cell surface, into the haemocoel. From the haemocoel, the virus infects secondary target organs, such as neuronal ganglia and salivary glands, resulting in a fully disseminated infection (Jennings & Mellor, 1987). Once an insect has acquired a disseminated infection it remains infectious for the rest of its live, but is unable to pass on the virus transovarially (Jones & Foster, 1971; Fu, 1996). The ovary appears to be protected from infection by the existence of an ovarial sheath (Fu, 1996).

Instead of infecting the midgut cells and emerging from their abluminal side, virus particles may also reach the haemocoel directly through gaps in the monolayer midgut cell sheet. This phenomenon is referred to as a “leaky gut” syndrome and may be of particular significance in non-vector Culicoides species, since it allows virus particles to bypass refractory midgut cells. Once virus has reached the haemocoel, it will usually be replicated in secondary target organs such as neuronal ganglia and salivary glands. It may then be secreted with the saliva in subsequent blood-meals. The potential significance of the “leaky gut” syndrome is highlighted by the fact that an increase in the larval rearing temperatures of C. nubeculosus, a northern European species not considered to be a competent vector of BTV or AHSV, leads to a significant number of insects developing a fully disseminated infection (Mellor et al., 1998) (Wittmann, 2000). This suggests that a change in climatic conditions, with a significant rise in temperatures, could “create” new vector species. Bypassing of the midgut cells is also aided by the simultaneous infection of insects with virus and microfilariae (Mellor & Boorman, 1980).

In vector-competent Culicoides species, higher environmental temperatures accelerate virogenesis, resulting in higher infection rates and faster dissemination of infection (Wellby et al., 1996; Wittmann, 2000). This reduction of the time between ingestion of an infectious blood meal and the presence of virus in the saliva (EIP) potentially enhances vector capacity. However, high temperatures also tend to decrease
adult lifespan which has a diminishing effect on vector capacity (Wellby et al., 1996; Wittmann, 2000).

1.3.5 Barriers to orbivirus infection, dissemination and transmission in the insect

Even in vector-competent Culicoides species, such as the North American C. sonorensis for BTV or the African C. imicola for AHSV and BTV, only a proportion of insects are likely to be competent vectors for a particular virus. Failure of a virus to turn an insect into a transmissive vector can be due to a number of barriers, which are outlined in Figure 1-7 (page 40).

1. Failure to infect the midgut cells (mesenteronal infection barrier, MIB).
2. Failure to spread from the infected midgut cells into the haemocoel, (mesenteronal escape barrier, MEB).
3. Failure to disseminate through the haemocoel following release from the midgut cells through the basal lamina (dissemination barrier, DB).
4. Failure to infect the salivary gland from the haemocoel (salivary gland infection barrier, SGIB).
5. Failure to be released from the salivary gland into the saliva during blood-feeding (salivary gland escape barrier, SGEB).
Figure 1-6: Schematic diagram of arbovirus replication in a competent vector
(adapted from Mellor et al., 2000)

Figure 1-7: Hypothesised barriers to the arbovirus infection of haematophagous insects
(adapted from Mellor et al., 2000)

*Barriers shown to be present in the C. sonorensis-AHSV/BTV; MIB-Mesenteronal Infection Barrier; MEB-Mesenteronal Escape Barrier; DB-Dissemination Barrier; TOTB-Transovarial Transmission Barrier; SGIB-Salivary Gland Infection Barrier; SGEB-Salivary Gland Escape Barrier.
1.3.6 The model vector system *C. sonorensis* (= *C. variipennis sonorensis*)-
AHSV/BTV

Terminology and laboratory colonies

To facilitate vector competence studies of BTV in its North American field
vector, then known as *C. variipennis* and comprising a total of five subspecies
(Holbrook *et al.*, 2000), a laboratory colony of the subspecies *C. v. sonorensis* was
established in the USA in 1957 (Jones, 1957). Eggs obtained from the American 000
colony spawned a new colony at Pirbright which has been successfully maintained
without further introductions since 1967 (Boorman, 1974). In 1995, insects from this
colony were selected for or against susceptibility to oral infection with AHSV-9 (Wellby
*et al.*, 1996). Selection took place over three generations of females and the vector-
competent colony was designated PIRB-s-3 (Wellby *et al.*, 1996). Both colonies have
been maintained without further selection or additions since then and have been named
the VC (vector-competent) = PIRB-s-3 and the NVC (non vector-competent) for the
purpose of this work. The uncertain taxonomic status of *C. v. sonorensis*, the subspecies
most closely associated with orbivirus transmission in North America, has been
reflected in the literature by the synonymous use of the term “*C. variipennis*” in various
vector competence studies (Jones & Foster, 1974; Boorman *et al.*, 1975). The term “*C.
variipennis* complex” has also been used.

Based on isozyme electrophoresis and morphological differences, there is now
strong evidence for the existence of at least three genetically distinct groups within this
complex (Tabachnick, 1990; Holbrook *et al.*, 2000). The absence of intermediate forms
in an environment of high sympatry (habitat-sharing) seems to justify the proposition of
three species within the old *C. variipennis* complex, which are now called *C. sonorensis*
(elevated from subspecies status), *C. variipennis* and *C. occidentalis* (Holbrook *et al*.,
2000). The Pirbright colonies originated from populations that are now designated as *C.
sonorensis* and this term is used throughout this thesis.

Genetic control of vector competence

The ability to select and maintain laboratory colonies with varying susceptibility
rates for AHSV and BTV from a parent colony of *C. sonorensis* shows that the
expression of the barriers to virus infection and dissemination is controlled by the insect
genotype (Jones & Foster, 1974; Wellby et al., 1996). It has been suggested that a single gene locus confers resistance to arbovirus infection when occupied by the dominant allele, while the presence of the recessive allele or alleles imparts susceptibility, the degree of which is under polygenic control (Jones & Foster, 1974). Evidence for the existence of a single gene locus with a strong influence on resistance and susceptibility has also been found by Tabachnick, although the presence of further gene loci controlling resistance and susceptibility could not be ruled out. In relation to the single gene locus, the maternal genotype appears to define the F1 phenotype while the paternal genotype is passed on to the F2 generation in a dominant fashion (Tabachnick, 1991).

The existence of a MIB and a MEB has been demonstrated in the C. sonorensis-BTV system (Jennings & Mellor, 1987; Fu et al., 1999). The existence of a mechanism hindering virus spread through the haemocoel has also been shown for this system and implicates the fat body as a defence organ (Fu et al., 1999). While there is evidence that no SGIB or SGEB exists in Culicoides species (Jennings & Mellor, 1987; Fu et al., 1999), these barriers have been shown to exist in various mosquito-arbovirus systems (Hardy et al., 1983; Hardy, 1986). The combination of these data suggests that the hereditary factors described above affect the midgut escape or infection barriers, the dissemination barrier, or a combination of them.

**Effects of virus titre and virus strain**

The titre of the infectious blood meal has been shown to have an effect on the proportion of insects which develop a persistent infection (infection rate) and it has been postulated that an "appropriate" titre of the blood meal will result in infection rates approaching the insect-defined "susceptibility rate" while significantly lower blood meal titres will result in much lower infection rates (Jones & Foster, 1974). The same work also suggests that infection rates close to the insect's genetically defined susceptibility rate can only be achieved by the "appropriate" virus. As a protein involved in virus exit from infected cells (see 1.2.3, page 26), it can be speculated that NS3/3a has a direct effect on the MEB.
1.4 Project objectives

The infection of an insect vector by an arbovirus and the dissemination of that virus through the vector's body are complex processes. The factors that determine whether infection is successful and results in transmission of the virus are multiple. They can be classed into insect-defined, virus-defined and environmental factors. However, these factors often exert their effect in an indirect fashion and modify each other. This and the difficulty involved in working with vector insects explain why at present, the mechanisms controlling the various barriers to virus dissemination in the insect are poorly understood. Yet, global climate-change and the ever-increasing international movement of humans, livestock and goods potentially aid the spread of many human, animal and crop pathogens and their arthropod vectors. The recent and current outbreaks of bluetongue in the Mediterranean basin, which have affected several countries that have never before recorded the disease, are typical examples of this. Consequently, control measures aimed at arboviral infections, in particular, will become more important. Clearly, the design and implementation of effective control strategies will benefit from a good understanding of the mechanisms that control the vector status of an insect.

The first aim of this project was to assess the effect of variation in AHSV segment 10 on oral infection rates and vector competence in the model vector C. sonorensis. It was hypothesised that, because of its involvement in virus release from the cell, NS3/3a (encoded by segment 10) may be necessary for virus dissemination from the cell and variation in this protein may thus modulate the mesenteronal escape barrier of the insect. Two reassortant viruses that differ only in virus genome segment 10 were available to help test this hypothesis. These two reassortant viruses, AHSV-A79 and AHSV-A790, were previously produced in the Orbivirus group by Dr. R. O'Hara and allowed her to identify a modifying role for NS3/3a in virus virulence in mice. Two laboratory colonies of C. sonorensis, previously selected for and against vector competence for AHSV-9, were used to study the role of NS3/3a in virus dissemination within adult insects. To aid in the interpretation of the data obtained, it was necessary to determine the AHSV titre that indicates a fully disseminated infection in the insect vector. This is an important benchmark for the present work and for future studies on the C. sonorensis-AHSV model system.
The second aim of this project was to assess the vector competence of two field vectors, *C. imicola* and *C. bolitinos*, for various AHSV strains including the reassortant viruses. The effect of variation in virus genome segment 10 on wild insects was tested and its relevance to the epidemiology of AHS considered. The susceptibility of the two field vectors to three highly attenuated AHSV strains was also examined. The attenuation process used to produce these strains was identical to the attenuation procedure used for the production of a previous generation of commercial, live attenuated virus vaccines. The safety of such live attenuated virus vaccines with regard to virus spread by insect vectors, potential genome reassortment and reversion to virulence is discussed.

The third aim of this project was to develop techniques for the characterisation of mesenteronal infection and escape barriers. These techniques were then used to investigate the susceptibility of *Culicoides* midgut cells to AHSV infection from the luminal and the abluminal sides. Results, when using a susceptible and a non-susceptible population of the vector species *C. sonorensis* and the orally refractory species *C. nubeculosus*, are compared.

The fourth aim of this project was to collect preliminary data leading towards an understanding of the cellular mechanism at the basis of the effect that NS3/3a has on virus dissemination. These experiments included cell-release assays carried out in a *C. sonorensis* (KC) cell line.
2 Materials and methods

2.1 Standard solutions

Tris-Acetate/EDTA electrophoresis buffer (10x TAE)

For one litre:

- 48.8 g Tris base
- 11.42 g Glacial acetic acid
- 20.0 ml 0.5 M EDTA pH 8.0

Electrophoresis sample buffer (5x ESB)

- 0.32 M Tris-HCl (pH 6.8)
- 10% (w/v) SDS
- 10% β-Mercaptoethanol
- 0.02% (w/v) Bromophenol Blue
- 15% (v/v) Glycerol

Tris-glycine buffer

- 200 mM Glycine
- 25 mM Tris-HCl (pH 8.8)
- 0.1% (w/v) SDS

K-MES

0.5 M MES equilibrated to pH 6.3 with KOH and sterilised by filtration (200 nm syringe filter). Stored at −20°C.

Luria-Bertani (LB) medium

For 1 litre:
• 10 g Bacto tryptone
• 5 g Bacto yeast
• 10 g NaCl

in distilled water, adjusted to pH 7.0 with NaOH and autoclaved.

2 M Magnesium ions (Mg$^{2+}$)

1 M MgCl$_2$ + 1 M MgSO$_4$

SOB medium

For one litre:
• 20 g Bacto tryptone
• 5 g Bacto yeast
• 0.584 g NaCl (10 mM)
• 0.186 g KCl (2.5 mM)

in distilled water, adjusted to pH 7.0 with NaOH and autoclaved.

Tris-EDTA buffer (10x TE buffer)

For one litre:
• 100 ml 1M Tris pH 8.0
• 20 ml 0.5 M EDTA pH 8.0

Transformation buffer (TFB)

For one litre:
• 7.4 g KCl (100 mM)
• 8.9 g MnCl$_2$.4H$_2$O (45 mM)
• 1.5 g CaCl$_2$.2H$_2$O (10 mM)
• 0.8 g HACoCl$_3$ (10 mM)

filtered and stored at 4°C
### Solutions for DNA minipreparation

<table>
<thead>
<tr>
<th>Solution I</th>
<th>Solution II</th>
<th>Solution III</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-HCl (pH 8.0)</td>
<td>25 mM Tris pH 8.0</td>
<td>3 M KOAc (pH 4.8)</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>0.2 M NaCl + 1% SDS</td>
<td>for 100 ml:</td>
</tr>
<tr>
<td>50 mM glucose</td>
<td>90.2 M NaCl</td>
<td>60 ml 5 M KOAc</td>
</tr>
<tr>
<td>5 mg/ml lysozyme</td>
<td>5 mM EDTA pH 8.0</td>
<td>11.5 ml glacial acetic acid</td>
</tr>
</tbody>
</table>

#### TNE buffer
- 50 mM Tris pH 8.0
- 0.2 M NaCl
- 5 mM EDTA pH 8.0

#### Sodium/Sodium citrate buffer (20x SSC solution)
- 175.3 g NaCl
- 88.2 g sodium citrate
- adjust to pH 7.0 with 10 N solution of NaOH
- adjust volume to 1 l with H₂O

#### Denhardt’s solution (100x)
For 100 ml 100x solution:
- 2 g Ficoll
- 2 g Polyvinylpyrrolidone (PVP)
- 2 g Bovine serum albumin (BSA)

#### Prehybridisation buffer
For 20 ml buffer:
- 10 ml formamide
- 5 ml 20x SSC solution
- 2 ml 100x Denhardt's solution
- 0.2 ml 10% SDS
- 0.2 ml salmon sperm DNA at 10 mg/ml (Sigma, boiled and iced immediately before use)
- 2.6 ml H$_2$O

**Hybridisation buffer**

For 40 ml:

- 20 ml formamide
- 10 ml 20x SSC solution
- 0.8 ml 100x Denhardt's solution
- 0.4 ml 10% SDS
- 0.4 ml salmon sperm DNA at 10 mg/ml (Sigma)
- 1% (w/v) glycine
- H$_2$O to 40 ml

**ELISA coating buffer (pH 9.6)**

- 0.1 M carbonate/bicarbonate buffer

**ELISA blocking buffer**

- 0.05% Tween 20 in PBS
- 5% (w/v) skimmed milk powder (Marvel)

**Western blot transfer buffer**

- 14.4 g Tris base
- 3.0 g glycine
- 200 ml methanol
- H$_2$O to 1000 ml
Western blot blocking buffer

- 0.1% Tween
- 5% Marvel milk powder
- TBS (below) to 1000ml

Tris buffered saline (TBS) 10x stock solution

- 48.8 g Tris base
- 160 g NaCl
- H₂O to 2 l, adjusted to pH 7.6 with HCl

2.2 Cells and viruses

2.2.1 Cell culture

Mammalian cells

BHK-21 cells were provided by the Arbovirology group at IAH Pirbright and were identical with those used for diagnostic purposes. They were therefore regularly tested for their sensitivity against AHSV and BTV. Cells were grown as monolayers in 175 cm² flasks until 90% confluent, using Dulbecco’s modified Eagles medium (DMEM) with 5% foetal calf serum (FCS).

Insect cells

The Culicoides sonorensis (KC) cell line was developed from two-day-old embryos of C. sonorensis (Wechsler et al., 1989). These cells were obtained from Dr W. Wilson in February 2001. They were grown in Schneider’s insect medium containing 15% FCS at 28°C. C. sonorensis cells have repeatedly been shown to grow orbiviruses without showing CPE (Wechsler et al., 1989; Fu et al., 1999; Hutchinson, 1999).

2.2.2 Stock viruses

All stock viruses were provided by courtesy of Dr R. O’Hara (IAH Pirbright), Dr. S. Zientara, AFSSA, Maisons Alfort, or the OIE Regional Reference Laboratory for AHS and BT at Pirbright. All viruses except AHSV-4 (Morocco), AHSV-A79 and
AHSV-A790 were originally isolated from South African horses and were originally obtained from the OIE World Reference Laboratory at Onderstepoort, South Africa.

**South African AHSV strains**

Virus designation, date of isolation and passage history of the virus strains that were originally obtained from South Africa are shown in Table 2-1.

<table>
<thead>
<tr>
<th>Virus name(^1)</th>
<th>Designation(^2)</th>
<th>Date of freeze-drying</th>
<th>Passage No.(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3wt</td>
<td>13/63</td>
<td>4/12/87</td>
<td>MB3; BHK9</td>
</tr>
<tr>
<td>6wt</td>
<td>39/63</td>
<td>23/7/75</td>
<td>MB3; BHK8</td>
</tr>
<tr>
<td>8wt</td>
<td>10/62</td>
<td>25/11/63</td>
<td>MB3; BHK9</td>
</tr>
<tr>
<td>3att</td>
<td>L</td>
<td>29/6/60</td>
<td>+BHK6</td>
</tr>
<tr>
<td>6att</td>
<td>114</td>
<td>29/6/60</td>
<td>+BHK6</td>
</tr>
<tr>
<td>8att</td>
<td>18/60</td>
<td>27/4/76</td>
<td>+BHK7</td>
</tr>
</tbody>
</table>

1 - The number indicates serotype; wt stands for minimal virus attenuation ("wild type") and att denotes heavily attenuated strains that underwent ≥100 passages in neonatal mouse brain.

2 - Number of specimen received by the laboratory/year of isolation. This information was not available for the virus isolates 3att and 6att.

3 - BHK(n) represents the number of passages in BHK-21 cells and MB(n) symbolises the number of passages in neonatal mouse brain carried out at IAH Pirbright (excluding the passages during attenuation of att strains).

**The reassortant viruses AHSV-A79 and AHSV-A790**

Reassortant viruses were produced by Dr R. O’Hara in the Pirbright Orbivirus group (O’Hara, 1994; O’Hara *et al.*, 1998). They were produced by co-infection of BHK-21 cells with AHSV-3att and AHSV-8wt and underwent at least three plaque purifications. The parental origins of their genome segments were subsequently determined by SDS-PAGE and, in the case of segment 9, by sequencing (O’Hara *et al.*, 1998). Reassortant virus strains A790 and A79 were found to differ in segment 10 only (Table 2-2, page 51). All differences in biological properties between them must therefore be due to this change. These viruses underwent up to 10 passages in BHK-21 cells.
Table 2-2: Derivation of genome segments in the two reassortant viruses AHSV-A790 and AHSV-A79

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Derivation of genome segments</th>
<th>Virulence in Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>3att (v)</td>
<td>vvvvvvv</td>
<td>virulent</td>
</tr>
<tr>
<td>8wt (a)</td>
<td>aaaaaaaaa</td>
<td>avirulent</td>
</tr>
<tr>
<td>A790</td>
<td>aaaaaavavv</td>
<td>intermediate</td>
</tr>
<tr>
<td>A79</td>
<td>aaaaaavava</td>
<td>avirulent</td>
</tr>
</tbody>
</table>

1- The A in the reassortant virus names indicates that the majority of their genome segments is derived from the avirulent (in mice) parent AHSV-8wt; the numbers behind it specify which genome segments are derived from the other parent strain AHSV-3att (O’Hara et al., 1998).

2- Genome segments derived from the AHSV-3att are designated a and segments derived from AHSV-8wt are designated v.

a, v- Highlighting the variation between A790 and A79 in genome segment 10.

AHSV-4 (Morocco)

This virus strain was originally isolated during the 1989-1991 AHS outbreak in Morocco and supplied by courtesy of Dr. S. Zientara, Maisons Alfort, France. It was exclusively used for immunofluorescence studies and underwent 4 passages in BHK-21 cells at IAH Pirbright.

2.2.3 Virus propagation

175 cm² flasks containing a 90% confluent monolayer of BHK-21 cells had their growth medium removed and were inoculated with 100 µl of virus solution, containing AHSV at circa 4 log_{10} TCID₅₀. After approximately 30 min incubation at room temperature the virus was removed and maintenance medium was added. Cells were then incubated at 37°C until full CPE was observed, usually after 48 to 72 h. At this point, the tissue culture fluid was removed and centrifuged for 5 min at 700 g to separate it from suspended cells. The supernatant was then removed and stored at 4°C. Its titre was determined using the virus infectivity assay (below) before it was used for experiments.
2.2.4 Virus infectivity assay

Ten fold dilution series of samples were prepared in DMEM. At least four 100 µl samples of the each dilution were added to individual wells of a 96-well tissue culture plate (Nunc), containing a 90% confluent monolayer of BHK-21 cells in 100 µl DMEM (1% FCS, 1% of 100 iu/ml penicillin and 100 µl/ml streptomycin). 100 µl DMEM was added instead of virus to one row per plate to act as a negative control. Plates were incubated at 37°C for at least seven days. From day three, plates were examined daily for the presence of CPE. Examination was carried out using optical microscopy. Inconclusive readings were verified by an indirect sandwich, antigen detection ELISA (below). Virus infectivity was calculated using the method of Spearman and Kärber and expressed as log_{10}TCID_{50}/ml (Kärber, 1931):

\[
\frac{TCID_{50}}{volume} = X_0 - \frac{d}{2} + \frac{d}{n} \sum X_i
\]

\(X_0\) - highest dilution at which all wells were positive (showed CPE)

\(d = \log_{10}\) of the dilution step (\(\log_{10}10 = 1\))

\(n\) - number of wells (replicates) per dilution tested

\(\sum X_i\) - sum of all positive wells above and including \(X_0\).

**Example:**

<table>
<thead>
<tr>
<th>Log_{10} virus dilution</th>
<th>Number of positive wells/number of replicates per dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4/4</td>
</tr>
<tr>
<td>1</td>
<td>4/4</td>
</tr>
<tr>
<td>2</td>
<td>1/4</td>
</tr>
<tr>
<td>3</td>
<td>0/4</td>
</tr>
</tbody>
</table>

\[
TCID_{50}/100\mu l = 1 - \frac{1}{2} + \frac{1}{4} \times 5 = 1.75 \quad \Rightarrow \quad TCID_{50}/ml = 2.75
\]

2.2.5 Antigen detection ELISA

The indirect sandwich ELISA for the detection of AHSV antigen was carried out according to a method developed by the regional AHSV reference centre at IAH Pirbright (Hamblin *et al.*, 1991). Antisera were raised against purified ISVP (AHSV serotype 9) and titrated prior to being used in tests to determine the optimum dilution
for each of them. Antisera and all other reagents were provided by courtesy of C. Hamblin at IAH Pirbright.

50 µl of rabbit hyperimmune antiserum, diluted 1/1000 with coating buffer, were added to each well of a Dynatech ELISA plate and adsorbed in a humidity chamber for 16 h at room temperature. The plate was washed three times with PBS and 50 µl aliquots of the sample were added to the wells. The plate was then incubated on an orbital shaker at 37°C for 1 h and washed with PBS as before. 50 µl of guinea-pig antiserum was added to each well at a 1/400 dilution in blocking buffer and the plates were incubated on an orbital shaker and washed with PBS as before. 50 µl rabbit anti-guinea-pig serum conjugated to horse radish peroxidase (Sigma) was added to each well at a dilution of 1/4000 in blocking buffer and the plates were incubated and washed as before. 50 µl chromagen/substrate solution (ortho-phenylene diamine at 0.4 mg/ml containing 0.05% H2O2 (30%) were added to each well. The reaction was stopped after 15 min with 50 µl 1.25 M sulphuric acid per well. Plates were read spectrophotometrically at 492 nm using a “Titretek Multiscan Plus” ELISA reader.

2.2.6 Serum neutralisation test

AHSV strains were serotyped against reference sera obtained from the OIE Regional Reference Laboratory at IAH Pirbright, following an established protocol used by this centre. The test is based on the formation of antibody-antigen complexes when a virus strain is incubated with its homologous antiserum. This reaction is serotype-specific and dependent on the antigen-antibody ratio. When the antibody concentration is sufficiently high then all virus particles will be bound in complexes and will therefore not infect monolayers of susceptible BHK-21 cells. This apparent reduction in virus titre is visualised by the absence of CPE, which in contrast will be present at the same virus concentration if incubated with non-homologous serum. For an AHSV test strain to be identified as one of the nine serotypes, its apparent titre (calculated as for the virus infectivity assay above) has to be reduced by ≥ 2 log10 (100 fold) by the appropriate serum. Cross-reactions are known to occur between the following serotypes: 1-2, 3-7, 5-8 and 6-9 (Lord et al., 1998).

10% horse serum was heat-inactivated by 30 min incubation at 56°C and added to microtitration plates at 100 µl/well. Test virus solution was diluted ten fold in tissue
culture fluid and added to these wells at concentrations of $10^{-1}$ to $10^{-7}$ of the original tissue culture supernatant. Plates were then incubated at 37°C and 5% CO₂ for one hour, 4°C overnight and again at 37°C and 5% CO₂ for one hour. This was followed by the addition of 50 µl BHK-21 cells in tissue culture supernatant with 3% FCS at $10^5$ cells/ml. Plates were then incubated at 37°C and 5% CO₂ for seven days and examined for the presence of CPE daily from day three. Every sample and dilution was tested in quadruplicates using sera against all nine serotypes of the virus. Controls were included for each virus strain tested and included one control of BHK-21 cells in tissue culture/FCS only, with no serum and no virus added. This was to ensure that the cells used in the test were viable. A second control contained cells and titrated virus but no serum. This was in effect a virus infectivity assay as described in section 2.2.4 and served to established the virus titre present in the sample and the reduction in AHSV infectivity caused by each reference serum. A third control contained cells and titrated virus but AHSV-seronegative (normal) horse serum. This controlled the quality of the reference sera and measured their non-specific binding of AHSV.

2.3 **Insects**

2.3.1 **Origin and identity**

The identity of the insects used for this work is summarised in Table 2-3 (page 55). *C. sonorensis* and *C. nubeculosus* were obtained from the Pirbright laboratory colonies of these insect species. *C. imicola* and *C. bolitinos* were collected nightly between February and March 2000 near livestock in South Africa, using down-draught suction light traps equipped with 8 W UV light tubes (Venter et al., 1998). Catching sites for *C. imicola* and *C. bolitinos* are shown in Figure 2-1 (page 55). Mosquito netting was placed around the traps to prevent the capture of larger insects. Midges were collected into plastic beakers with crumpled paper providing shelter from the down-draught of the trap. Insects were collected each morning before sunrise and transferred into unwaxed cardboard cups with mesh tops. They were then maintained at 23.5±1°C with a 10% sucrose solution medicated with penicillin, gentamycin and streptomycin. They were starved for 24 h before blood-feeding to increase their feeding rate.
Table 2-3: Culicoides insects used in this work

<table>
<thead>
<tr>
<th>Insect species (subgenus); designation</th>
<th>Vector status</th>
<th>Natural distribution</th>
<th>Origin of insects in experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. sonorensis</em> (Monoculicoides), formerly <em>C. variipennis sonorensis</em>; vector-competent colony (VC), previously named PIRB-s-3</td>
<td>BTV field vector, AHSV model vector with high oral susceptibility</td>
<td>For the USA and Canada, see Figure 1-1, page 13; also in Mexico</td>
<td>Taken from the wild in 1957 and colonised in Denver, USA; since 1967 maintained at Pirbright, UK, selection of females for/against oral susceptibility to AHSV-9 over 3 generations</td>
</tr>
<tr>
<td><em>C. sonorensis</em> non-competent colony (NVC)</td>
<td>BTV field vector, AHSV model vector with low oral susceptibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. imicola</em> (Avaritia)</td>
<td>Major AHSV field vector</td>
<td>Africa, S and SE Asia, some parts of S Europe (Figure 1-2, page 14)</td>
<td>Live wild catches at Onderstepoort near Pretoria (25°29'S, 28°11'E; 1219 m a.s.l.) and Clarens (eastern Free State at 28°32'S, 28°25'E; 1631 m a.s.l.), S. Africa</td>
</tr>
<tr>
<td><em>C. bolitinos</em> (Avaritia)</td>
<td>AHSV field vector, probably of regional significance</td>
<td>Southern Africa</td>
<td>Live wild catches at Clarens, S. Africa (see above)</td>
</tr>
<tr>
<td><em>C. nubeculosus</em> (Monoculicoides)</td>
<td>Usually refractory to oral infection with AHSV or BTV</td>
<td>Palaearctic</td>
<td>Taken from the wild in Hertfordshire in 1969 and maintained at Pirbright since 1971</td>
</tr>
</tbody>
</table>

Figure 2-1: Catching sites for *Culicoides* insects in South Africa
(A) Onderstepoort, (B) Clarens
2.3.2 Oral infection and maintenance of *C. sonorensis* and *C. nubeculosus* insects

*C. sonorensis* and *C. nubeculosus* were orally infected at the Pirbright laboratory following a method described previously (Mellor *et al.*, 1974), with the chick skin membrane being replaced by a parafilm membrane (Figure 2-2, page 57). Three-day-old adult insects were used in all experiments unless stated otherwise. They were allowed to feed on a 1:1 mixture of AHSV solution (infected BHK-21 tissue culture supernatant) and fresh heparinised horse blood for approximately 30 min. After feeding, fully engorged females were selected under a CO2 induced anaesthesia and placed in a meshtop, waxed card pillbox (max. 150 individuals/box). They were maintained at 25°C and provided daily with a 10% sucrose solution, supplemented with 100 iu/ml penicillin, 100 µl/ml streptomycin and 2.5 µl/ml fungizone. At the end of the nine-day extrinsic incubation period, survivors were selected under CO2 anaesthesia and stored individually in Eppendorf tubes at -70°C until assayed unless stated otherwise.
Figure 2-2: Oral feeding apparatus for *C. sonorensis* and *C. nubeculosus*
(reproduced with permission from Wittmann, 2000)

Figure 2-3: Oral feeding apparatus for *C. imicola* and *C. bolitinos"
2.3.3 Oral infection and maintenance of *C. imicola* and *C. bolitinos* insects

Insects were collected and maintained until blood-feeding as described in section 2.3.1. They were blood-fed through a one-day-old chick skin membrane using a previously described method (Venter *et al.*, 1991; Figure 2-3). Defibrinated, AHSV-negative and AHSV-seronegative horse blood was used to produce a suspension of 10% AHSV solution (in tissue culture supernatant) and 90% horse blood. A sample of the blood meal was taken before and after blood-feeding, stored at -70°C and later assayed to ensure that there was no significant drop in virus titre during the course of the blood meal. Feeding lasted 30 min and was carried out at 23.5°C, 50-70% relative humidity and approximately 1% daylight. The blood meal had a temperature of 37°C. Engorged females were immobilised at -10°C and then transferred to a chill table. Fully engorged *Culicoides* were selected and placed in an unwaxed cardboard cup with a fine mesh top. They were maintained at 23.5°C with a cotton wool pad soaked in a 10% medicated sucrose solution as before the blood meal. After 10 days, surviving *Culicoides* were identified to species level on the chill table and individually stored in Eppendorf tubes at -70°C until assayed.

2.3.4 Intrathoracic (IT) inoculation of *C. sonorensis* and *C. nubeculosus* insects

Insects were inoculated into the thorax with AHSV using a procedure described by Boorman (Boorman, 1975). Briefly, a semiautomatic device was used (Figure 2-4, page 59). It is based on the controlled release of air pressure to force the inoculum into the body of the insect and allows constant volumes to be inoculated. The applied air pressure and the time for which pressure is applied can both be controlled individually and compensate for variation in aperture size between needles.
2.3.5 Grinding of insect samples

Insect samples to be assayed for the presence of virus were defrosted, individually ground with motor-driven plastic pestles (Anachem) in 1.5 ml Eppendorf tubes and suspended in 1 ml of tissue culture fluid. Antibiotics and an antifungal agent were added at 100 iu/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. Samples were then assayed as described in section 2.2.4, page 52.

2.4 Electrophoresis

2.4.1 Agarose gel electrophoresis

Agarose gels consisted of 0.8% agarose or low melting point agarose in TAE buffer. Agarose was melted in a microwave and ethidium bromide (10 mg/ml) was diluted 1:10,000 into the solution before gels were poured. Electrophoresis was carried out in TAE buffer at 80-120 V for 30-40 min. Samples were visualised under UV light.

2.4.2 SDS-PAGE

Samples were mixed 1 in 5 in 5x ESB. Protein samples were denatured with a freeze-boil cycle. Analysis by 11% SDS polyacrylamide gel electrophoresis (PAGE) was carried out using a method adapted from Laemmli (1970). Resolving gels (1.5 mm
thick, 15 cm wide, 16 cm long) were run with Tris-glycine buffer at up to 200 V/60 mA until the dye front reached the bottom of the resolving gel. Gels were then used for RNA cross-hybridisation experiments (see sections 2.5.13 and 2.5.14, page 64) or dried onto Whatman 3 MM filter paper on a vacuum dryer (Hoefer) at 60°C for 4h and exposed to X-ray film.

2.5 **Biochemical and molecular techniques**

2.5.1 **RNA extraction**

Virus RNA was extracted following a modified protocol published in 1981 (Clarke & McCrae, 1981). Alternatively, RNA was extracted using Trizol (Life Technologies) according to the manufacturer's instructions.

2.5.2 **Reverse transcription of virus genome segment 10**

Reverse transcription (RT) from dsRNA to cDNA was carried out using modified standard protocols (Sambrook et al., 1989). 3 µg of genomic virus dsRNA was mixed with 1 µl of each primer (at 25 pmol/µl) in H₂O (total volume 10 µl), denatured at 95°C for 5 min and immediately transferred to chilled methanol (stored at -70°C) to facilitate primer-binding before renaturing of dsRNA strands would set in. The RNA-primer mix was then incubated with 2 µl methyl-mercaptop-hydroxide at room temperature for 10 min. This reducing reaction was stopped with 1 µl 0.7 M β-mercaptop-ethylamine. After a further 5 min the whole reaction mix was added to a premix of 1 µl RNAsin, 5 µl 10x RT reaction buffer, 2.5 µl DTT, 2 µl 20 mM dNTP mix and 24.5 µl H₂O (total volume 48 µl). Heating of this mix to 37°C was followed by the addition of 2 µl reverse transcriptase and incubation at 37°C for 40 to 60 min. Reverse transcriptase RT reaction buffer were supplied by Life Technologies. Primers used were those published by Martin et al. (1998): (5' GTTTA AATTA TCCCT TGTC...3') upstream and (5' GTAAG TCGTT ATCCC GGCT...3') downstream and were supplied by Cruachem or MWG.

2.5.3 **PCR amplification of genome segment 10**

cDNA was amplified by using standard protocols (Sambrook et al., 1989). 5 µl of the RT reaction product (above) was used in the following reaction mix:
- 10 µl 10x reaction buffer
- 1 µl DTT
- 2 µl dNTP mix
- 2 µl MgCl₂ (50 mM)
- 2 µl primer 1 (25 pmol/µl)
- 2 µl primer 2 (25 pmol/µl)
- 5 µl RT reaction product
- 75 µl H₂O
- 1 µl taq polymerase

The mix was vortexed, spun down to the bottom of the tube and overlayed with mineral oil. The PCR reaction included an initial 5 min denaturation at 95°C, followed by 30 cycles of 1 min denaturing at 95°C, 1 min annealing at 55°C and 1 min extending at 72°C. The last cycle was followed by an extra 7 min extension time at 72°C. The reaction was carried out with taq polymerase and reaction buffer supplied by Roche Pharmaceuticals. Primers used were the same as those used in the RT reaction.

2.5.4 Alternative RT-PCR protocol

RT-PCR reactions were also carried out using a kit supplied by Amersham Pharmacia. The kit consists of ready-to-use beads that include all the reagents required for the reverse transcription and the amplification steps of the reaction.

The primer-template mix was heated to 95°C for 3 min, placed on ice for 2 min and then added to the beads which had been reconstituted with milli-Q water. Primers were the same as in section 2.5.2 above. Reaction conditions were those described in 2.5.2 and 2.5.3, with the PCR reaction immediately succeeding the RT reaction. The main advantages of this protocol are better standardisation of the reaction, a reduction in pipetting (= potential for error or contamination) and a combination of the RT and PCR steps in one tube, combined in one program on the thermocycler.
2.5.5 **Purification of cDNA**

PCR products were excised from a LMP (low melting point) agarose gel and extracted using phenol/phenol-chloroform/chloroform (Sambrook *et al.*, 1989).

2.5.6 **Ligation of cDNA**

DNA fragments were ligated into plasmids with the pGEM-T Easy Vector System I (Promega), using T4 DNA Ligase and 2x Rapid Ligation Buffer.

2.5.7 **Transformation of *E. coli***

25 ml of SOB medium was mixed with 0.5 ml of 1 M Glucose and 250 µl of 2 M Mg^{2+}. The resulting growth medium was inoculated with three to five colonies of XL I Blue cells and the culture shaken overnight at 37°C. 25 ml of SOB medium, 0.5 ml of 1 M Glucose and 250 µl of 2 M Mg^{2+} was inoculated with 100 µl of that overnight culture and shaken at 37°C until it reached an OD_{600} of 0.4-0.6. The culture was then placed on ice for 10 min, transferred to a universal and spun for 10 min at 100 g and 4°C.

The pellet was gently resuspended in 12.5 ml of ice-cold transformation buffer (TFB), placed on ice for 10 min, spun for 10 min at 100 g, resuspended in 2 ml TFB and placed on ice for 5 min. Dimethyl formamide (DMF) was added to 3.5% (70 µl) and the solution swirled and placed on ice for 5 min. 70 µl β-mercapto-ethanol in 10 mM K-MES (5.2 µl β-mercapto-ethanol + 100 µl K-MES) was added and the tubes placed on ice for a further 10 min. Another 70 µl DMF was added, the solution swirled and aliquoted into 200 µl quantities.

10 µl ligation mix was added to the fresh competent cells, mixed by inverting and placed on ice for 30 min. The cells were then heat shocked at 42°C for 45 seconds, added to 800 µl SOB medium and shaken for 1 h at 37°C.

The transformed cells were spread on LB ampicillin IPTG (Isopropylthio-β-D-galactoside) & X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) agar plates and incubated overnight at 37°C. Positive (white) colonies were picked with cocktail sticks, cultured in 10 ml LB media containing ampicillin and used for DNA minipreparation.
2.5.8 Minipreparation of DNA

An overnight culture of bacteria transformed with plasmid DNA was centrifuged at 100 g for 10 min. The supernatant was stored at 4°C for maxipreparations, the pellet resuspended in 0.1 ml solution I and placed at room temperature for 5 min. 0.2 ml solution II was added, the mixture inverted several times and left on ice for 10 min. 0.15 ml solution III was added, the tubes were vortexed and placed on ice for a further 5 min. Cell debris was removed by 5 min centrifugation at 1200 g and 4°C, followed by the addition of 300 µl isopropanol to the supernatant. After vortexing and 2 min centrifugation at 1200 g, the pellet was resuspended in 100 µl TE and the DNA precipitated with the addition of 10 µl 3 M sodium acetate in 330 µl ethanol and incubation on ice for 2 min. The DNA was pelleted at 1200 g for 5 min, resuspended in 50 µl TE and stored at -20°C.

2.5.9 Restriction endonuclease analysis

DNA minipreparations were used to check plasmids for the presence of correct-size inserts. These pGEM-T Easy plasmid digests were carried out using 1 µl EcoRI enzyme, 1 µl RNAse A, 2 µl 10x buffer H or Multi-Core (all provided by Promega) and 1 µg DNA in a reaction volume of 20 µl. Incubation for 1 h at 37°C was followed by agarose gel electrophoresis.

2.5.10 Maxipreparation of DNA

Midi- and Maxipreparations of DNA were carried out using QUIAGEN Midi and Maxi kits, following protocols provided by the manufacturer.

2.5.11 Sequencing of plasmid DNA

Plasmid DNA was sequenced using a cycle sequencing kit (ALFexpress AutoCycle Sequencing Kit, supplied by Pharmacia). Electrophoresis was carried out and results were processed on an ALFexpress automatic sequencing machine (Pharmacia). DNA sequences were exported into the sequence analysis programs GCG 10 and GeneDoc and analysed as described in section 2.6 (page 67).
2.5.12 RNA probe production

A RNA probe for cross-hybridisation was produced following a modified published protocol (Mertens et al., 1987). Approximately 1 µg of dsRNA was fragmented in 100 µl 0.1 M NaOH at 0°C. The reaction was stopped after 10 min with 15 µl 1 M acetic acid and 10 µl 1 M Tris-HCl (pH 8.0). The RNA was then precipitated, washed and dried as described in the RNA extraction protocol. $^{[32P]}$-labelling was carried out by adding 2 µl $[^{32P}]dATP$, 2 µl polynucleotide kinase, 86 µl H$_2$O and 10 µl 10x kinase reaction buffer, followed by 40 min incubation at 37°C (Sambrook et al., 1989). Labelled dsRNA was then separated from unincorporated label using a Sephadex G100 column and stored in TNE buffer at -20°C.

2.5.13 dsRNA blotting

Transfer of denatured dsRNA from the polyacrylamide gel to a Hybond-N transfer membrane was carried out using a 2117 MULTIPHOR II electrophoresis unit. Twelve pieces of Whatman 3 MM filter paper and the transfer membrane were all cut to gel size and thoroughly soaked in blotting buffer (2.93 g glycine, 5.81 g Tris, 0.375 g SDS in 1 l dH$_2$O) prior to assembly of the blotting unit. The gel was then placed between nine layers of filter paper on the anodic side and three layers of filter paper on the catodic side. Electrophoresis was carried out at 250 V, 25 W for 3.5 h.

2.5.14 Cross-hybridisation of AHSV dsRNA

Samples of AHSV genomic dsRNA were subjected to SDS-PAGE. Each lane A was loaded with approximately 3000 cpm of the AHSV genomic RNA, which had been $[^{32P}]pCp$-labelled with RNA ligase. This radio-labelled RNA served as a size marker for the unlabelled genomic dsRNA of the same AHSV strain that was loaded in each lane B at about 1 µg per lane.

The dsRNA was denatured by soaking the gel in 0.1 M NaOH for 30 min immediately after electrophoresis (24 h at 150 V). The gel was then washed in blotting buffer (section 2.5.13), blotting of dsRNA onto a Hybond-N transfer membrane (Amersham) was carried out as described in above and the dsRNA was crosslinked to the transfer membrane with a Spectrolinker XL-1000 (Spectrometers Corporation). The membrane was incubated in prehybridisation buffer for 4 h at 50°C and then incubated
for a further 16 h in the hybridisation solution/RNA probe mixture. The probe was boiled for 3 min immediately before addition to the preheated hybridisation buffer. Hybridisation was followed by four washes with 2x SSC containing 0.1% SDS at room temperature and two washes with 0.1x SSC containing 0.1% SDS at 58°C. Each wash lasted 15 min. The membrane was then dried and autoradiographed at -70°C. Alternatively, a phosphor imaging screen was used.

2.5.15 Conjugation of peptide to keyhole limpet haemocyanin (KLH) via SMCC

SMCC [Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carbonate, Pierce] contains an activated carboxyl group at one end which can react with amino groups, but also a maleimido group at the opposite end that reacts with the sulfhydryl group of cysteine residues. SMCC can therefore be used as a crosslinker between the peptide and carrier proteins such as KLH (Pierce). SMCC was reacted with the amino groups on the carrier protein: 150 µl KLH (10 mg/ml in dH2O) plus 100 µl SMCC (4 mg/ml in dH2O) at room temperature for 1h, followed by removal of unreacted crosslinker using a desalting column. 10 mg peptide was dissolved in 0.5 ml DMSO and 1.5 ml dH2O. 10 mg of the SMCC-activated KLH was added and the reaction mix incubated for 4h at room temperature. The solution was then dialysed overnight against PBS, using a Slide-a-Lyzer dialysis cassette (Pierce). The peptide-KLH conjugate was used to immunise guinea pigs and rabbits.

2.5.16 Sample fixation for immunofluorescent microscopy

KC cell monolayers on microscopy coverslips or freshly dissected Culicoides guts were incubated with 4% paraformaldehyde for 30 min (guts 45 min), washed with PBS three times and permeabilised in PBS plus 0.5% Triton X-100 for 10 (15) min. Samples were rinsed with PBS three times and stored at 4°C or processed for immunofluorescence microscopy immediately.

2.5.17 Staining protocol for immunofluorescent microscopy

Fixed samples were blocked with 1% BSA in PBS for 1 h. Primary antibodies were added to the blocking buffer and incubated with the samples for a further hour. This was followed by five washes with PBS and incubation with the appropriate secondary antibodies (Table 2-4) for 1 h. Three PBS washes were then followed by
sample mounting on microscopy slides in Vectashield mounting medium (Vector Laboratories, Burlingame, USA). Alternatively, samples were successively incubated for 30 min with one of Texas Red-Wheat Germ Agglutinin, Oregon Green-Phalloidin and TO-PRO-3 (Table 2-4). Samples were washed in PBS three times after each incubation and then mounted on microscopy slides. All incubations were carried out at room temperature.

Table 2-4: Fluorophore-conjugated secondary antibodies and stains used in immunofluorescence microscopy

<table>
<thead>
<tr>
<th>Antibody/Stain</th>
<th>Target</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Guinea Pig antibody 488 nm</td>
<td>Guinea Pig IgG</td>
<td>Green</td>
</tr>
<tr>
<td>Goat anti-Guinea Pig antibody 594 nm</td>
<td>Guinea Pig IgG</td>
<td>Red</td>
</tr>
<tr>
<td>Goat anti-Rabbit antibody 594 nm</td>
<td>Rabbit IgG</td>
<td>Red</td>
</tr>
<tr>
<td>Goat anti-Mouse antibody 594 nm</td>
<td>Mouse IgG</td>
<td>Red</td>
</tr>
<tr>
<td>Texas Red-Wheat Germ Agglutinin</td>
<td>Lectins in cell membranes</td>
<td>Red</td>
</tr>
<tr>
<td>Oregon Green-Phalloidin</td>
<td>Actin filaments</td>
<td>Green</td>
</tr>
<tr>
<td>TO-PRO-3</td>
<td>DNA, RNA</td>
<td>Blue</td>
</tr>
</tbody>
</table>

1 supplied by Molecular Probes, Eugene, USA

2.5.18 Western blots

Infected or uninfected cells were lysed with lysis buffer (Promega) 24 h (post infection (pi) and stored at -20°C if necessary. Lysates were then denatured at 100°C for 5 min and the proteins resolved by SDS-PAGE on mini-gels (Bio-Rad) in conjunction with a pre-stained broad range protein marker (New England Bio-labs) at 200 V and 400 mA until the dye front ran off the bottom of the gel. Proteins were transferred onto an Immobilon P membrane (Millipore) in transfer buffer (see section 2.1, page 45) at 100 V and 400 mA for 1 h. The membrane was placed into 10 ml blocking buffer (section 2.1) in a 50 ml Falcon tube and incubated overnight with constant rotation.

For development, the primary antibody was added to the tube and incubated for 1 h under constant rotation. The membrane was washed twice for 30 sec and then twice for 10 min in TBS/1% Tween. It was then incubated with the secondary antibody (15 μl anti-rabbit peroxidase-linked antibody, Amersham) in 10 ml blocking buffer for 1 h with constant rotation and washed as before. Blots were developed by incubating with
Luminol enhancer and Stable Peroxide (2.5 ml of each, SuperSignal, Pierce) for 1 min. Blots were immediately wrapped in Saran wrap and exposed to film.

2.6 **Nucleotide and protein sequence analysis**

Nucleotide sequences obtained with the ALF automatic sequencing machine were imported into GCG 10, aligned and exported into GeneDoc (version 2.6) as a multisequence file (MSF). Further alignment, translation into predicted protein sequences and comparison to other predicted protein sequences (imported from databases such as TrEMBL and SwissProt) were carried out in GeneDoc. The output was used to grow unrooted neighbour-joining trees in GCG 10, using the “distances” command. The resulting trees were edited in TreeView (version 1.6.6). GeneDoc files of protein sequences were searched for potential motifs using a PROSITE database of such motifs. Transmembrane arrangements of individual NS3 sequences were predicted using GCG 10 and the web-based programs TMHMMM, TMAP and HMMTOP (listed at http://ca.expasy.org/tools/#pattern).

2.7 **Statistical data analysis**

Datasets were tested for statistical significance between them using a Chi-Square ($\chi^2$) test. This test compares the number of positives and the number of negatives in a series of datasets to the numbers that would be expected to occur if the datasets belonged to the same entity. Differences between expected and real values receive a score and on the basis of these scores a $\chi^2$ value is calculated. At $p=0.05$, $\chi^2>3.84$ indicates a statistically significant difference at one degree of freedom ($d.f.=1$) and $\chi^2>7.81$ indicates statistical significance at $d.f.=3$. A $p$-value of 0.05 denotes a 5% chance that the conclusion of statistical significance reached by the test is incorrect. Lower $p$-values indicate a lower risk of an erroneous result, e.g. $p=0.01$ means 1% likelihood that the stated test result is incorrect. The degree of freedom is calculated as $n-1$ where $n$ is the number of datasets compared. For instance, for a comparison of infection rates between two populations of an insect species: $d.f.=2-1=1$. If more than two datasets are compared (e.g. four batches of an insect colony infected with four different viruses, $d.f.=3$) and the test indicates statistically significant differences between them, then significant differences may be found in one, more than one or all six pairwise comparisons of these four batches.
3 Characterisation of AHSV strains

3.1 Introduction

Virus characteristics, including sample origin, passage history, serotype and attenuation level are important for the correct interpretation of experimental results. This chapter focusses on the two reassortant viruses AHSV-A79 and AHSV-A790 because they are a very important tool for establishing the role of NS3/3a in AHSV infection of the insect vector.

AHSV-A790 and AHSV-A79 were produced in the Orbivirus group at IAH Pirbright by Dr. R. O'Hara (O'Hara, 1994; O'Hara et al., 1998). They were derived from the parent strains AHSV-3att and AHSV-8wt through co-infection of BHK-21 cells, followed by three plaque purifications. The origin of their 10 genome segments was determined by SDS-PAGE, taking advantage of the variation in migration speed of the dsRNA genome segments between the two parent strains. Since segment 9 of AHSV-3att co-migrates with that of AHSV-8wt, this segment was sequenced in each of the reassortant viruses (O'Hara et al., 1998). Origins and passage histories for the reassortant virus strains and all other viruses used in this work are shown in section 2.2.2 (page 49).

The first aim of this chapter was to confirm the identity of the two reassortant viruses. These two viruses and their parent virus strains AHSV-8wt and AHSV-3att were characterised using a serum neutralisation test (SNT), polyacrylamide gel electrophoresis (SDS-PAGE) and cDNA sequencing (section 2, page 45).

The second aim of this chapter was to identify motifs of potential biological significance in the NS3 and NS3a sequences and any differences in these regions between the two reassortant viruses. Such motifs include, for instance, ubiquitinase-binding motifs (see section 1.2.3, page 26), myristylation motifs (membrane anchors) and glycosylation sites. The presence of such motifs does not demonstrate that they are biologically active. However, differences in the presence or distribution of these and other motifs could account for different biological virus properties conferred by the two variants of the protein.
To identify potentially significant motifs, genome segment 10 cDNA sequences were translated into protein sequences and analysed as described in section 2.6 (page 67). Full NS3 sequences were analysed wherever possible. Therefore, this chapter does not collectively refer to the two proteins as NS3/3a, as do other sections of this thesis. NS3 proteins differ from the corresponding NS3a sequences by the presence of 10 to 11 additional, in-frame, N-terminal amino acid (aa) residues.

3.2 Results

3.2.1 Serum neutralisation test

The results of the SNT are shown in Table 3-1. The titre of AHSV-3att was reduced from $6.0 \log_{10} TCID_{50}/ml$ (titre with normal horse serum) to $3.0 \log_{10} TCID_{50}/ml$ with anti-serotype 3 serum, a reduction by $3.0 \log_{10} TCID_{50}/ml$. Other sera reduced the AHSV-3att titre by much smaller and insignificant margins, ranging from $0 \log_{10} TCID_{50}/ml$ (serotype 4) to $1.25 \log_{10} TCID_{50}/ml$ (serotype 7).

The titres of AHSV-A790, AHSV-A79 and 8wt were most heavily reduced by anti-serotype 8 serum. This serum caused a reduction in virus titre (compared to normal horse serum) of 3.25, 3.5 and 3.5 $\log_{10} TCID_{50}/ml$, respectively. Antisera to other serotypes reduced the virus titres by 0.25-1.0 $\log_{10} TCID_{50}/ml$ (AHSV-A790), 0.25-1.5 $\log_{10} TCID_{50}/ml$ (AHSV-A79) and 0.25-1.25 $\log_{10} TCID_{50}/ml$ (AHSV-8wt), with antiserum to serotype 5 causing the largest reduction in all three viruses.
Table 3-1: Serum neutralisation test with four AHSV strains

<table>
<thead>
<tr>
<th>Serum serotype</th>
<th>Normal horse serum</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-3att</td>
<td>6.0</td>
<td>5.5</td>
<td>5.5</td>
<td>3.0</td>
<td>6.0</td>
<td>5.25</td>
<td>5.25</td>
<td>4.75</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>AHSV-A790</td>
<td>6.0</td>
<td>5.25</td>
<td>5.25</td>
<td>5.75</td>
<td>5.5</td>
<td>5.0</td>
<td>5.5</td>
<td>5.75</td>
<td>2.75</td>
<td>5.25</td>
</tr>
<tr>
<td>AHSV-A79</td>
<td>7.0</td>
<td>6.0</td>
<td>6.25</td>
<td>6.5</td>
<td>6.75</td>
<td>5.5</td>
<td>6.0</td>
<td>6.25</td>
<td>3.5</td>
<td>6.0</td>
</tr>
<tr>
<td>AHSV-8wt</td>
<td>6.5</td>
<td>5.75</td>
<td>5.5</td>
<td>6.0</td>
<td>6.25</td>
<td>5.25</td>
<td>5.5</td>
<td>5.75</td>
<td>3.0</td>
<td>5.75</td>
</tr>
</tbody>
</table>

Ten-fold dilution series of AHSV strains were incubated with normal horse serum (negative control), or horse serum raised against one of the nine AHSV serotypes, in 96-well microtitration plates. BHK-21 cells were added and formed a monolayer at the bottom of the wells. Neutralisation of AHSV by homologous antibodies abolished viral CPE in the BHK-21 cells. CPE was looked for between days 3 and 7. Virus titres were calculated in log_{10} TCID_{50}/ml (Kärber, 1931). A virus titre reduction of at least 2 log_{10} TCID_{50}/ml against one serum compared to all others, indicated that the tested virus was homologous to this serum. Cross-reactions commonly occur between the following serotypes: 1-2, 3-7, 5-8 and 6-9 (Lord et al., 1998). Titres in red signify a titre reduction that indicates that the virus belongs to the serotype tested.

1-Negative control serum.

3.2.2 Polyacrylamide gel electrophoresis

SDS-PAGE was carried out, as described in section 2.4.2 (page 59), to confirm the origin of the genome segments in the reassortant viruses. Figure 3-1 A) (page 71) shows that segment 10 of AHSV-A790 migrated at the same speed as segment 10 of AHSV-3att, while AHSV-A79 segment 10 migrated at the same speed as AHSV-8wt segment 10. Figure 3-1 B) shows that AHSV-A790 and AHSV-A79 segments 1-6 and 8 migrated at the same rate as those of AHSV-8wt and that AHSV-A790 and AHSV-A79 segments 7 migrated at the same rate as that of AHSV-3att. Segment 9 migrated at the same speed in all four virus strains.
Figure 3-1: SDS-PAGE images of dsRNA genomes: Reassortant viruses AHSV-A790 and AHSV-A79; parent virus strains AHSV-3att and AHSV-8wt

A) This image shows that segments 10 of AHSV-8wt and AHSV-A79 (left) co-migrate, as do segments 10 of AHSV-A790 and AHSV-3att (right), (red arrows).

B) This gel contained more evenly matched amounts of AHSV-A79 and AHSV-A790 dsRNA and shows that these two virus strains are identical with AHSV-8wt in the migratory pattern of segments 1-6 and 8, but identical with AHSV-3att for segment 7. The identity of reassortant segment 9 cannot be established from this image but was previously established by sequencing (O’Hara et al., 1998). The identity of reassortant segment 10 is not obvious from B) due to insufficient separation (shorter running time) but is visible in A).
3.2.3 Sequencing of genome segment 10

Figure 3-2 (page 74) shows the alignment of predicted amino acid sequences for NS3 of reassortant and parent virus strains. These were obtained through cDNA sequencing of genome segment 10 from AHSV-A790, AHSV-A79, AHSV-8wt, or were retrieved from the EMBL database in the case of AHSV-3att (which had previously been sequenced in the Orbivirus group). Sequencing of AHSV-8wt did not result in a full-length segment 10 sequence and the predicted sequence of its NS3 therefore starts at aa residue 12. This corresponds to aa residue 1 for virus protein NS3a in this virus strain.

The figure illustrates that the two reassortant viruses AHSV-A790 and AHSV-A79 differ in 57 out of 217 aa residues of NS3, equivalent to 26% protein sequence variation. It also shows that AHSV-A790 NS3 is identical with AHSV-3att in 215 of 217 residues (>99% homology), while homology between AHSV-A79 and AHSV-8wt is >99.5% (205 of 206 residues). The type of NS3 found in AHSV-A79 and AHSV-8wt (α group, Figure 3-3, page 75) is very highly conserved at the N-terminal (appendix I, page 160), the region missing from the AHSV-8wt sequence.

Figure 3-2 shows potential myristylation, ubiquitin ligase-recruiting and glycosylation motifs. AHSV-3att and AHSV-A790 each possess two potential myristylation motifs near the NS3 N-terminal (aa 15 and aa 37), which are absent in AHSV-8wt and AHSV-A79. The latter two viruses each possess one potential myristylation site near the NS3 C-terminal (aa 211), which is absent in AHSV-3att and AHSV-A790. A further myristylation site and an ubiquitin ligase recruiting motif are conserved in NS3’s of all four viruses. NS3 of AHSV-A79 shows a potential N-glycosylation site at aa 9, which is absent in AHSV-3att and AHSV-A790 (with no AHSV-8wt sequence available for this domain). However, appendix I shows that this region is identical for all but one of the α-group NS3 available.

Figure 3-3 illustrates the differences in the NS3 amino acid sequences between the reassortant viruses and their parent strains, in the context of other available AHSV NS3 sequences. It confirms that the NS3 proteins of AHSV-A79 and AHSV-A790 cluster into different groups (α and β groups, respectively), alongside their parent strains. The figure also illustrates previous observations that the type of NS3 does not
always correlate with serotype (van Niekerk et al., 2001b). Isolates of AHSV-8 cluster into all three groups, while isolates of AHSV-1 cluster into two groups.

Figure 3-4 (page 76) shows the predicted configuration of NS3 in the cytoplasmic membrane for AHSV-A79 (α group) and AHSV-A790 (β group) together with a γ group, serotype 8 virus (ST8_Q64905; prediction method see section 2.6, page 67). It illustrates that NS3 of AHSV-A79 is likely to have two transmembrane regions, thus conforming to the model suggested by van Staden et al. (1995). In contrast, NS3 of AHSV-A790 is more likely to possess only one transmembrane region (as is ST8_Q64905). These protein configurations are representative for all NS3 sequences from the three clusters, with the one exception of ST4_DQB5. This virus strain belongs to the α group (Figure 3-3) but, in the model used, is equally likely to have two transmembrane helices (like other α group NS3’s) or only one transmembrane region (like all β and γ group NS3’s).
**Figure 3-2: NS3 protein sequence alignment of two reassortant viruses and their two parent strains**

Differences between NS3 sequences from parent and reassortant viruses are shown in **RED/BLUE**, potential ubiquitin ligase recruiting motifs are **GREEN**, potential myristylation motifs are **PINK** and a possible glycosylation motif is **GREY** (AHSV-A79, aa 9).

Segment 10 nucleotide sequences of virus strains AHSV-8wt, A79 and A790 were obtained by sequencing their RT-PCR products directly or after cloning as described in section 2.5, page 60. Segment 10 of AHSV-3att was sequenced previously in the Orbivirus group (Martin et al., 1998) and its sequence was obtained from the EMBL database (AJ007304). All sequences were aligned and translated into protein sequences using GeneDoc. Sequencing of AHSV-8wt did not result in a full-length sequence (first 11 residues are missing, sequence represents full NS3a sequence).

This figure shows >99% homology between the predicted protein sequences for AHSV-8wt and A79; and separately between AHSV-3att and A790.
Figure 3-3: Clustering of reassortant and parent virus NS3 sequences into α, β and γ groups of AHVS NS3

Unrooted neighbour-joining tree of AHVS NS3 protein sequences. Reassortant viruses and their parent strains are highlighted in RED (α group) or GREEN (β group). Protein sequences were obtained from SwissProt and TrEMBL databases, determined by DNA sequencing and translation (AHVS-A79, AHVS-8wt, AHVS-A790) or obtained from EMBL as a nucleotide sequence and translated (AHVS-3att, accession number AJ007304). For protein sequences obtained from databases, the serotype (ST) is given, followed by the accession number. Bar (bottom left) represents 1% variation. The tree was designed using the “distances” program in GCG 10.
Probable transmembrane, cytoplasmic and extracellular locations of NS3 regions were calculated in the program TMHMM (available at http://www.cbs.dtu.dk/services/TMHMM-2.0).

All three NS3 strains are highly likely (>90%) to have their N-terminal located in the cytoplasm (blue line), to have a transmembrane helix at aa 111/112-133/134 (red), followed by an extracellular domain (pink). This extracellular domain is predicted to stretch from aa 135 to 153 in AHSV-A79, followed by another transmembrane helix (aa 154-173, predicted with 64% certainty) and a cytoplasmic C-terminal. AHSV-A790 and ST8_Q64905 are likely (62% certainty) to have only one transmembrane helix, followed by an extracellular C-terminal.
3.3 Discussion

Identity of reassortant AHSV genome segments

The results of the SNT confirm that AHSV-3att is a serotype 3 virus (Table 3-1, page 70). In addition, the reduction in titre of this virus when titrated against AHSV serotype 7 antiserum was greater than with any other serotype (other than serotype 3). This is evidence of the well-documented cross-reactivity between these two serotypes (Lord et al., 1998). The virus strains AHSV-A790, AHSV-A79 and AHSV-8wt all typed as serotype 8 viruses (Table 3-1). In addition, for all three viruses, titre reduction was greater when titrated against anti-serotype 5 serum than against any of the remaining sera. Again, this is evidence of the cross-reactivity between AHSV serotypes 5 and 8. The experiment confirms the serological status of AHSV-3att and AHSV-8wt. For the reassortant viruses, it can be concluded that the serotype-defining VP2 and VP5, encoded by genome segments 2 and 6, are derived from the parent AHSV-8wt.

The results of the SDS-PAGE suggest that AHSV-A790 segment 10 is identical with AHSV-3att segment 10 and that the segment 10 of AHSV-A79 is identical with that of AHSV-8wt. The results also suggest that segments 1-6 and 8 of both reassortant viruses are derived from AHSV-8wt while their segment 7 is derived from AHSV-3att. Although all segment 9's run at the same speed and their origin therefore cannot be deduced from SDS-PAGE, segment 9 has previously been shown to be derived from AHSV-3att in both reassortant viruses by cDNA sequencing (O'Hara et al., 1998). The results are in keeping with previous SDS-PAGE results (O'Hara, 1994) and with the concept that the two reassortant virus strains differ in segment 10 only (as shown in Table 2-2, page 51).

The results of the sequencing of reassortant and parent virus genome segment 10's show conclusively that segment 10 of AHSV-A79 is derived from AHSV-8wt, while segment 10 of AHSV-A790 is derived from AHSV-3att. This also confirms previous results (O'Hara, 1994). The variation of the AHSV-A790 NS3 sequence from the AHSV-3att in two aa residues could be due to point mutations that have occurred since the reassortant was derived from its parent strains, could be a gel reading error or could result from the RT-PCR reaction. Similarly, the single difference in NS3 sequence between AHSV-A79 and AHSV-8wt may have the same origin. Sequencing did not reveal the 11 N-terminal aa residues of AHSV-8wt NS3, but the high degree of
conservation of this region in all α-group sequences (appendix I) strongly suggests that it is also conserved in this virus, and thus likely to be identical with AHSV-A79.

Potential motifs and transmembrane regions in NS3 protein sequences

Comparison of NS3 protein sequences was preferred over analysis of cDNA sequences because only variation in protein sequences is likely to lead to differences in the biological properties of the two reassortant viruses. This comparison also allowed motifs of potential biological significance to be identified.

The identification of identically located ubiquitinase binding motifs (PPxY, at aa 25), in both reassortants and their two parent strains, means that the presence of this motif cannot explain biological differences between these viruses. However, the conservation of this motif in all α- and γ-group NS3's and the majority of β-group NS3's suggests that it is of biological significance. It may be that, in common with identical motifs in the retrovirus gag protein (see section 1.2.3, page 26), it may facilitate virion exit from the cell. An additional P(T/S)AP ubiquitinase binding motif, present in all available γ-group sequences at aa 30, might further enhance virion budding from the host cell. A combination of PPxY and P(T/S)AP motifs in close proximity on a chimaeric retrovirus gag protein was found to enhance budding of VLPs (Strack et al., 2000).

The identification of a myristylation motif at aa 60 in both reassortant viruses and all analysed sequences of other AHSV’s (located at aa 59 in γ-group NS3) confirms the previous finding of van Niekerk et al. (2001b) and suggests that this binding site for the membrane anchor myristylic acid may be essential for protein targeting to the cell membrane. Membrane anchors are usually but not exclusively found at the N-terminal of the protein, for instance in HIV-1 (Zhou et al., 1994). Additional myristylation motifs are present in different locations in the two reassortants and are highly conserved within each NS3 cluster (see Figure 3-2, page 74 and appendix I, page 160). It is noteworthy that all possible myristylation motifs are located in predicted cytosolic protein domains, which is required for their functioning as membrane anchors. Interestingly, the NS3 of ST4_Q9DQB5, which is the only α-group protein without a C-terminal myristylation motif, is also the NS3, in this group, with the lowest likelihood (50%) of possessing a cytosolic C-terminal.
The potential N-terminal N-glycosylation site found in AHSV-A79 is present in all α-group NS3’s but this region is strongly predicted to be cytosolic (indicating that glycosylation of this site is very unlikely). All other identified potential glycosylation sites are also very strongly predicted to be cytosolic or (in the γ-group NS3’s) to be part of the transmembrane helix. Glycosylation of any of the analysed NS3 variants is therefore highly unlikely. If this is so then it means that no known motifs are present in the N-terminal of NS3, which would be absent in NS3a. Such motifs, had they been present, could have indicated differences in protein function between NS3 and NS3a.

Potential glycosaminoglycan-binding sites were identified in AHSV-A790, AHSV-3att and many other β-group NS3’s. Binding of glycosaminoglycans is a characteristic of secretory proteins and the significance of this motif in NS3 is unclear. A large number of theoretical phosphorylation sites is present in all NS3 variants but these are too numerous to discuss.

In the absence of techniques for the successful rescue of orbivirus particles from the introduction of cDNA molecules into host cells, the significance of the potentially important motifs that have been discussed above could be explored by cloning and mutating AHSV NS3’s, and expressing the mutants in a baculovirus expression system together with all other, wild-type AHSV proteins. Reduced or abolished budding of VLPs from the cell could then be related to the mutations of NS3. More simply, mutated NS3’s could be expressed in a baculovirus system or, using vaccinia virus, they could be transfected into host cells. The cellular location of NS3 could then be visualised with anti-NS3 antibodies. Expression of NS3 with an attached Green Fluorescent Protein (GFP) would make it possible to follow the path of protein translocation after synthesis using live fluorescent microscopy. The effects of specific mutations could then be studied.

The different transmembrane arrangement of the two reassortant viruses is predicted with only with approximately 2:1 likelihood by the program TMHMM. Very similar results were obtained with the program GCG 10. Other programs, including the web-based TMAP or HMMTOP, produced different numbers of possible transmembrane helices (programs available at http://ca.expasy.org/tools/#pattern). All programs did, however, predict more transmembrane regions, and therefore a different membrane arrangement, for AHSV-A79 compared to AHSV-A790. It is therefore
possible that this different arrangement, perhaps in conjunction with the different number and location of myristylation motifs, is responsible for different biological properties of the two viruses in mammalian and insect host systems.
4 Culicoides sonorensis vector competence for AHSV

4.1 Introduction

AHSV is endemic in many parts of sub-Saharan Africa and causes a disease of high mortality in horses. Its main vector is C. imicola, a small haematophagous biting midge which occurs in most parts of Africa, large parts of Asia and some parts of southern Europe, where it has probably been spreading northward over the last two decades (Rawlings et al., 1998). Outbreaks of AHS in Europe occurred in 1966 in Spain (Diaz Montilla & Panos Marti, 1968) and in 1987-1990 in the Iberian peninsula (Rodriguez et al., 1992; Mellor, 1993; Mellor, 1994). As a result of these outbreaks an effort was made to predict areas at risk from AHSV.

The ability of AHSV to be transmitted and consequently to cause disease in the field depends on the availability of suitable insect vectors. Accurate prediction of at-risk areas therefore requires, among other things, a knowledge of vector capacity (i.e. the ability of the insect vector population to transmit the pathogen to the vertebrate host) and of how this is affected by variables in the insect, the virus strain and the environment (section 1.3.3, page 37). Vector competence (i.e. the ability of vector insects to become infected with the pathogen and transmit it to a suitable vertebrate host after the extrinsic incubation period, EIP) is one of the variables which determine vector capacity and is itself characterised by factors that can be classed as virus-defined, insect-defined and environmental.

This chapter concentrates on the effects of insect-controlled factors (such as age and previously selected susceptibility to AHSV-9) and virus-controlled factors (such as variation in virus genome segment 10) on the dissemination of AHSV in C. sonorensis and consequently on C. sonorensis vector competence. C. sonorensis is the North American field vector of BTV (Jones et al., 1981) and is a well characterised model vector of AHSV (Boorman et al., 1975; Mellor et al., 1975; Wellby et al., 1996). Two laboratory colonies of this species are maintained at IAH Pirbright. They were selected for high vector competence (VC colony) and low vector competence (NVC colony) for AHSV-9 in 1995 (Wellby et al., 1996) and have been maintained without further selection since then (section 1.3.6, page 41). C. sonorensis is used as a model because it
has thus far proved impossible to establish laboratory colonies of the field vector C. imicola (Mellor et al., 2000).

The first objective of this chapter was to establish the post-incubation virus titre that indicates a fully disseminated AHSV infection in C. sonorensis. A fully disseminated infection indicates infection of the salivary glands and that virus transmission is possible during subsequent blood meals on susceptible mammals. Knowledge of this titre is necessary to assess the effects of variation in insect-defined and virus-defined factors on virus dissemination within the insect and on vector competence. The BTV titre that indicates such a fully disseminated infection in the vector C. sonorensis is known to lie between 2.5 and 3.0 log10 TCID50/fly (Jennings & Mellor, 1987; Fu, 1996). These experiments, carried out by the Pirbright Arbovirology group, were based on virus transmission across a membrane during blood-feeding and release of virus during artificially induced salivation, respectively. The experiments described here were designed to obtain a measure of insect infectivity to mammalian hosts. Therefore, only methods based on the detection of live virus were considered as proof of virus presence in blood meals, saliva or body tissues. Methods for the identification of viral protein or RNA, such as ELISA, immunofluorescence microscopy, PCR or nuclear probes, although in some cases more sensitive, were considered unsuitable because of their ability to detect non-infectious viral proteins or RNA. Because of the complex nature of the experiments, three different methods were used to show fully disseminated infection in the insect:

1) Transmission of virus during membrane-feeding.

2) Artificial induction of salivation.

3) Virus infectivity assay in insect heads.

1) Detection of virus transmitted during membrane-feeding was selected as the method that most closely resembles virus transmission from the infected insect to a susceptible vertebrate host. Membrane-feeding through parafilm is a well established technique and has also been used in this chapter for infecting C. sonorensis and C. nubeculosus by the oral route (see 2.3.2, page 56).

2) Salivation was artificially induced by the application of Malathion (malathion 500 g/l; Murphy Chemical Co., Wheathamstead, Hertfordshire). Malathion contains the organophosphate diethyl-(dimethoxythiophosphorylthio)succinate (C10H19O6PS2) and
inhibits cholinesterase. The subsequent accumulation of the neurotransmitter acetylcholin at cholinergic synapses of the peripheral and central nervous system has various effects across the body. One of these effects is the overstimulation of the salivary glands and excessive salivation. The amount of virus secreted during Malathion-induced salivation, therefore, may not reflect the quantities released during blood-feeding. The presence of virus in the saliva will, however, prove that the virus has disseminated throughout the insect body and has infected the salivary glands.

3) Previous observations indicate that salivary gland infection or exit barriers do not exist in Culicoides species (Fu et al., 1999; Mellor et al., 2000), i.e. if the virus has replicated in secondary target organs such as the neuronal ganglia of the head, it will also have replicated in the salivary glands and will be secreted with the saliva during blood-feeding (section 1.3.5, page 39). Therefore, detection of live virus in the insect head was chosen as an indicator of full virus dissemination within the insect. Similar approaches have been taken before, for instance the recovery of dengue virus from mosquito legs as an indicator of virus dissemination in Aedes aegypti (Turell et al., 1987).

The experiments described in this chapter were carried out at 9 days post infection (dpi) unless stated otherwise. This EIP was chosen because C. sonorensis have been shown to develop maximum titres after an EIP of nine days (Wellby et al., 1996). To confirm that virus detected at 9 dpi is not a residue from the infectious blood meal, insects from the infection-refractory species C. nubeculosus (Table 2-3, page 55) were fed an infectious blood meal and assayed at 9 dpi in the same way as C. sonorensis. Assuming that the gut lumina of the two haematophagous species C. sonorensis and C. nubeculosus represent similar environments (both belong to the subgenus Monoculicoides), the absence of residual virus from C. nubeculosus at 9 dpi would indicate an inability of the virus to survive for this period in either Culicoides species, unless virus replication takes place. AHSV-8wt was used for the experiments in this chapter described so far, since it achieved the highest infection rates of all AHSV strains in C. sonorensis.

The second objective of this chapter was to determine whether susceptibility to AHSV varies with the age of the insect at the time of ingestion of an infectious blood
meal. This is particularly important for the standardisation of vector-competence studies and the comparison of the results of different workers. This experiment is based on an incidental observation in the early stages of this project, which is described in the next paragraph.

When experiments to establish infection rates in both C. sonorensis colonies with the reassortant viruses were started, insects were fed infectious blood meals at either two or three days of age. This terminology denotes the time span from placing mature pupae into pillboxes to the time of the blood-feeding. Most insects emerged during the first day in the pillbox. However, a small proportion usually hatched later and consequently were younger than the terminology would suggest. On one occasion, when blood-feeding two-day-old insects from the NVC colony with AHSV-A79, it was found that only a small proportion (less than 10%) of females had fully engorged. This particular batch of insects contained an unusually large number of teneral (i.e. very young) adults. Because of the poor feeding rate, the blood-feeding of a second batch of insects from the same colony and of the same age was delayed by 24 h. This allowed a comparison of the infection rates of two- and three-day-old insects under otherwise identical experimental conditions. Infection rates were determined using the methods described in sections 2.3.5 and 2.2.4 (pages 59 and 52). Disseminated-infection (DI) rates were calculated as the percentage of assayed insects that developed a fully disseminated infection, and would therefore be competent vectors.

A third objective of the work described in this chapter was to re-evaluate the levels of vector competence of the two C. sonorensis colonies. The data obtained provide an estimate of the colonies’ genetic stability since selection (i.e. over a period of several years). They also indicate whether selection for or against vector competence for an AHSV-9 strain lead to similar changes in the vector competence for other AHSV strains. To achieve this aim, insects from the VC C. sonorensis colony were infected orally as described in section 2.3.2 (page 56). Batches of insects were infected with one of the reassortants AHSV-A790 or AHSV-A79, or with one of the parent virus strains AHSV-3att or AHSV-8wt (section 2.2.2, page 49). To evaluate the vector competence of the NVC colony of C. sonorensis, batches of insects from this colony were also infected with one of AHSV-A790, AHSV-A79, AHSV-3-att or AHSV-8wt. Blood meal titres were identical with those used in the VC colony. Infection rates for all insect batches
were determined and DI rates calculated as described for the second objective of this chapter, above. The proportion of virus-positive insects that registered a fully disseminated infection was also determined for all insect batches.

To assess the stability of the two insect colonies in terms of their susceptibility to infection by the four virus strain, the experiment was repeated after an interval of approximately two years. On this occasion, the virus assay's sensitivity was increased ten-fold by assaying not only the first (and subsequent) ten-fold dilutions, but also the original, undiluted solution of 1 ml tissue culture media, containing one ground-up insect. This step reduced the minimum detectable virus titre from 1.75 (experiment I) to 0.75 (experiment II) log10 TCID50/insect. Titres of the blood meals ranged from 5.75 to 6.5 log10 TCID50/ml.

The fourth and arguably the most important objective of the work described in this chapter was to assess whether variation in AHSV genome segment 10 could affect virus dissemination throughout the body of the insect vector and, consequently, its vector status. Genome segment 10 codes for the small non-structural protein NS3/3a, which is involved in virus exit from the cell (Hyatt et al., 1993). Following oral ingestion by the insect and successful replication in the midgut cells, NS3/3a could affect virus release from the insect’s midgut into the haemocoel. Detection of a significant effect of genome segment 10 might indicate that variation in this segment represents adaptation to different insect vector species or populations. Such a finding would help to explain the observed high degree of variation in this genome segment. The data used to address these questions originate from the experiments used to re-examine the vector competence of the VC and NVC colonies (described above). Infection rates and DI rates were compared between the two reassortant viruses AHSV-A790 and AHSV-A79, which differ in segment 10 only.

The fifth aim of this chapter was to verify a personal communication by Dr. E. Wittmann from the Pirbright Arbovirology group that AHSV-6wt does not infect C. sonorensis by the oral route. This observation raised the question whether this virus strain is capable of infecting individuals of this species by any route and, if it is not, what is the mechanism that prevents the virus from establishing an infection in the
insect host via the oral route. AHSV-6wt readily replicates in both BHK-21 and KC cells. The latter are an embryonic tissue culture cell line derived from C. sonorensis. To confirm that C. sonorensis is resistant to oral infection with AHSV-6wt, insects from the VC colony were fed an infectious blood meal as before, containing AHSV-6wt at 6.5 log_{10} TCID_{50}/ml. Infection rates were determined as described before. To establish whether AHSV-6wt can successfully replicate in the insect if the gut barriers MIB and MEB (section 1.3.5, page 39) are bypassed, further C. sonorensis flies were inoculated intrathoracically with AHSV-6wt as described in section 2.3.4 (page 58).

4.2 Methods

Transmission of virus during membrane-feeding

C. sonorensis from the VC colony were given an infectious blood meal containing AHSV-8wt at a final concentration of 6.5 log_{10} TCID_{50}/ml. Approximately 100 engorged females were selected and incubated for nine days at 25°C as described in section 2.3.2 (page 56). Sucrose solution was withdrawn at 7 dpi to encourage subsequent blood-feeding. Survivors were then placed individually in 0.5 ml Eppendorf tubes that had had their bottoms cut off and replaced with parafilm (Figure 4-1, page 87). These tubes were positioned in the wells of microtitration plates containing 100 µl heparinised horse blood at 37°C. The bottom of the wells contained a 90% confluent monolayer of C. sonorensis (KC) cells (section 2.2.1, page 49). These cells have been found to be highly susceptible to infection with AHSV and were found to grow well in heparinised horse blood. The microtitration plates containing the preheated blood and the Eppendorf tubes with midges inside were incubated at 37°C for 1 h. Insects were then removed and stored at -70°C until assayed. 100 µl Schneider's insect medium was added to each well and the plates incubated at 29°C for seven days. Since KC cells do not show CPE, 100 µl supernatant was then transferred to BHK-21 cells to assay for the presence of AHSV as described in section 2.2.4 (page 52).
Figure 4-1: Membrane-feeding of individual *C. sonorensis* for the detection of AHSV transmission into a blood meal

**Artificial induction of salivation**

The experiment was conducted following a protocol that had previously been established for *C. sonorensis* (Boorman, 1987) and was subsequently used to establish the titre that indicates a fully disseminated infection of *C. sonorensis* with BTV (Fu, 1996). *C. sonorensis* from the VC colony were orally infected with AHSV-8wt (6.5 log_{10}TCID_{50}/ml) and fully engorged females were incubated for nine days at 25°C. 100 surviving midges were then individually placed on double-sided sticky tape in a Petri dish with their ventral side facing upward. A glass capillary tube was used to apply approximately 1 µl of 0.01% Malathion to the ventral abdomen. Over the following 20 min, saliva was collected from the probosces of salivating midges with very fine capillary tubes. The tubes were stored in tissue culture medium at -70°C until assayed for AHSV.

**Virus infectivity assay in insect heads**

*C. sonorensis* insects from the VC colony were orally infected with AHSV-8wt (6.75 log_{10}TCID_{50}/ml) and incubated at 25°C as described in section 2.3.2 (page 56). After an EIP of nine days, survivors were anaesthetised with CO₂ and their heads
separated from their bodies with dissecting needles. While the insect bodies were stored and assayed as described for entire insects in sections 2.3.5 (page 59) and section 2.2.4 (page 52), the heads were processed immediately. Individual heads were ground in 20 µl tissue culture fluid between the frosted surfaces of two microscopy slides. The resulting suspension was made up to 100 µl with tissue culture medium and assayed for the presence of virus in a single well of a microtitration plate (section 2.2.4, page 52).

4.3 Results

4.3.1 AHSV titre that indicates dissemination of infection in C. sonorensis

Transmission of virus during membrane-feeding

72 female C. sonorensis survived the nine-day EIP and were used in the transmission experiment. Only two of these midges then visibly blood-fed across the parafilm membrane, although some other individuals seemed to probe the membrane. No virus was detected in any of the 72 blood meal residues when tested for AHSV released by insects during blood-feeding. The lowest virus detection level was 0.75 log_{10} TCID_{50}/ml. To ascertain that the failure to transmit virus across the membrane was not due to an absence of infectivity in the insects, 21 of the 72 insects were ground up and assayed for AHSV, including the two visibly engorged midges. Virus was detected in 12 of these insects (infection rate: 57%) and the maximum titre detected was 3.5 log_{10} TCID_{50}/fly. The two blood-fed insects both proved to be virus-negative at the detection level of 0.75 log_{10} TCID_{50}/fly).

Artificial induction of salivation

Salivation could only be observed in five out of 100 insects. No AHSV was detected in any of the saliva samples collected (detection level: 0.5 log_{10} TCID_{50}/sample). Assay of the insects for AHSV showed that over 50% of insects were infected and titres ranged from 0.75 to 3.5 log_{10} TCID_{50}/fly (data not shown). Three of the five insects that visibly salivated were infected with virus titres of ≥3.0 log_{10} TCID_{50}/fly, the other two were virus-negative.
Virus infectivity assay in insect heads

The virus assay of insect heads and bodies shows that insect bodies containing less than $2.5 \log_{10} \text{TCID}_{50}$ were never associated with the presence of virus in the insect head while titres of $3.0 \log_{10} \text{TCID}_{50}$ or higher were consistently associated with virus presence in the head (Table 4-1). Titres of $2.5$ and $2.75 \log_{10} \text{TCID}_{50}$ were in most cases associated with an infected insect head.

Table 4-1: Dissemination of AHSV-8wt in C. sonorensis (VC colony)

<table>
<thead>
<tr>
<th>Virus titre in the insect body</th>
<th>Number of insect bodies</th>
<th>Number of virus-positives in the corresponding insect heads</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.25</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.75</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2.25</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>2.75</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3.25</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3.5</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Three-day-old insects were given an infectious blood meal of AHSV-8wt (6.75 $\log_{10} \text{TCID}_{50}$/ml) and incubated for 9 days at 25°C. Insect heads were separated from the bodies and both were stored at -70°C. Bodies were individually assayed for AHSV (minimum detection level: $0.75 \log_{10} \text{TCID}_{50}$/fly) and grouped according to their virus titres (column 2). Insect heads were assayed for AHSV and virus-positives were grouped according to the titres of their corresponding bodies (column 3).

Oral susceptibility of C. nubeculosus to AHSV-8wt – a control experiment

The results in Table 4-2 (page 90) show that adult females of the species C. nubeculosus, which is orally refractory for AHSV infection, do not contain detectable levels of AHSV at nine days after ingestion of a blood meal containing infectious virus. Since this C. nubeculosus has repeatedly been shown not to replicate AHSV following oral ingestion, this result indicates that non-replicating AHSV does not survive in Culicoides for nine days pi.
Table 4-2: Susceptibility of adult *C. nubeculosus* to oral infection with AHSV-8wt

<table>
<thead>
<tr>
<th></th>
<th>0 dpi</th>
<th>9 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number positive/number assayed</td>
<td>5/5</td>
<td>0/28</td>
</tr>
<tr>
<td>Range (log_{10}TCID(_{50}/fly)</td>
<td>3.0-3.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Three-day-old insects were given an infectious blood meal of AHSV-8wt (6.75 log_{10}TCID\(_{50}/ml) and incubated at 25°C for 9 days. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log_{10}TCID\(_{50}/fly).

4.3.2 Effect of insect age on susceptibility to oral infection with AHSV

The infection rates of the two insect batches are shown in Table 4-3 (page 91). The infection rates in the two-day-old insects were higher than in the three-day-old midges, but this difference was not of statistical significance (χ² = 2.680, p = 0.101, d.f. = 1). Nevertheless, in the two-day-old group there were significantly more insects with a high virus titre indicating the presence of a disseminated infection (DI rate; χ² = 12.448, p < 0.001, d.f. = 1). A titre of $>=2.5 \log_{10}TCID_{50}/insect$ was used as an indicator of the presence of a fully disseminated infection. This value results from the experiments described in section 4.3.1 and is discussed in section 4.4, page 97. The number of insects with a disseminated infection as a proportion of all infected was also significantly higher (χ² = 8.316, p = 0.004, d.f. = 1) in the two-day-old group. As a consequence of the considerable differences in infection rates between two and three day old insects, all vector competence studies were carried out with three-day-old insects. Using two-day-old insects throughout was not feasible because feeding rates were very variable and often too low to allow the determination of infection rates.
Table 4-3: Effect of insect age on oral infection and dissemination in *C. sonorensis* infected with AHSV-A79

<table>
<thead>
<tr>
<th>Insects infected at two days of age</th>
<th>Insects infected at three days of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number positive/number tested</td>
<td></td>
</tr>
<tr>
<td>(Infection rate IR in percent)⁴</td>
<td>12/27 (44%)</td>
</tr>
<tr>
<td></td>
<td>19/70 (14%)</td>
</tr>
<tr>
<td>Number with disseminated</td>
<td></td>
</tr>
<tr>
<td>infection/number tested (DI rate in percent)⁴</td>
<td>8/27 (30%)</td>
</tr>
<tr>
<td></td>
<td>3/70 (4%)</td>
</tr>
<tr>
<td>Number with disseminated</td>
<td></td>
</tr>
<tr>
<td>infection/number positive (percent)⁴</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td></td>
<td>3/19 (16%)</td>
</tr>
</tbody>
</table>

Insects from the NVC colony were given an infectious blood meal of AHSV-A79 (6.75 log<sub>10</sub> TCID<sub>50</sub>/ml) and incubated at 25°C for 9 days. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log<sub>10</sub> TCID<sub>50</sub>/fly).

1- Values rounded up or down to nearest integer

DI rate: Disseminated-infection rate
4.3.3 Infection rates of reassortant viruses and their parent virus strains in *C. sonorensis*

*Experiment I*

The data presented in Table 4-4 and Figure 4-2 (page 93) show a significantly higher IR achieved with AHSV-A79 than with AHSV-A790 in the VC colony ($\chi^2=14.081$, $p<0.001$, $d.f.=1$). There is no significant difference between any of the four virus strains in the NVC colony ($\chi^2=7.606$, $p=0.055$, $d.f.=3$). The same data also show that there are significant differences between the VC and the NVC colonies in regard of their IRs with AHSV-A79 ($\chi^2=31.552$, $p<0.001$, $d.f.=1$) and AHSV-8wt ($\chi^2=17.072$, $p<0.001$, $d.f.=1$), with the IRs being higher in the VC colony. However, AHSV-A790 exhibits no statistically significant difference in its ability to infect the VC and the NVC colonies ($\chi^2=3.504$, $p=0.061$, $d.f.=1$). Neither does AHSV-3att ($\chi^2=0.458$, $p=0.498$, $d.f.=1$).

DI rates (Table 4-4) show highly significant differences between VC and NVC colonies for AHSV-8wt ($\chi^2=20.762$, $p<0.001$, $d.f.=1$) and AHSV-A79 ($\chi^2=30.181$, $p<0.001$, $d.f.=1$), but the difference is only just significant for AHSV-A790 ($\chi^2=6.546$, $p=0.011$, $d.f.=1$). DI rates are higher with the VC colony than with the NVC colony for all three viruses. VC and NVC colonies do not differ significantly in their response to infection with AHSV-3att ($\chi^2=2.941$, $p=0.086$, $d.f.=1$). Between the two reassortant viruses, there is a statistically significant difference in the VC colony (AHSV-A79>AHSV-A790; $\chi^2=11.672$, $p=0.001$, $d.f.=1$) but not in the NVC colony ($\chi^2=1.123$, $p=0.289$, $d.f.=1$).

The number of insects with a disseminated infection as a proportion of all virus-positive midges does not differ significantly between the reassortant virus strains in the VC colony ($\chi^2=0.183$, $p=0.669$, $d.f.=1$) or the NVC colony ($\chi^2=0.114$, $p=0.735$, $d.f.=1$).
Table 4-4: Infection prevalences of two reassortant viruses and their parent strains in two colonies of *C. sonorensis* – Experiment I (1999)

<table>
<thead>
<tr>
<th>AHSV- strain</th>
<th>Vector-competent (VC) colony</th>
<th>Non-competent (NVC) colony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number positive/number tested</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Infection rate in percent)¹</td>
<td>(16%)</td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>8/49</td>
<td>32/101</td>
</tr>
<tr>
<td>A790</td>
<td>(16%)</td>
<td>(32%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>34/54</td>
<td>90/143</td>
</tr>
<tr>
<td>(63%)</td>
<td>(63%)</td>
<td>(21%)</td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>37/179</td>
<td>25/121</td>
</tr>
<tr>
<td>A790</td>
<td>(21%)</td>
<td>(21%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>27/54</td>
<td>69/143</td>
</tr>
<tr>
<td>(50%)</td>
<td>(48%)</td>
<td>(9%)</td>
</tr>
<tr>
<td>Number with disseminated infection/number tested (DI rate in percent)¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>1/49</td>
<td>24/101</td>
</tr>
<tr>
<td>A790</td>
<td>(2%)</td>
<td>(24%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>27/54</td>
<td>69/143</td>
</tr>
<tr>
<td>(50%)</td>
<td>(48%)</td>
<td>(9%)</td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>17/179</td>
<td>9/121</td>
</tr>
<tr>
<td>A790</td>
<td>(9%)</td>
<td>(7%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>3/70</td>
<td>14/70</td>
</tr>
<tr>
<td>(4%)</td>
<td>(20%)</td>
<td></td>
</tr>
<tr>
<td>Number with disseminated infection/number positive (percent)¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>1/8</td>
<td>24/32</td>
</tr>
<tr>
<td>A790</td>
<td>(12%)</td>
<td>(75%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>27/34</td>
<td>69/90</td>
</tr>
<tr>
<td>(79%)</td>
<td>(77%)</td>
<td>(46%)</td>
</tr>
<tr>
<td>AHSV- 3att</td>
<td>17/37</td>
<td>9/25</td>
</tr>
<tr>
<td>A790</td>
<td>(46%)</td>
<td>(36%)</td>
</tr>
<tr>
<td>A79 8wt</td>
<td>3/10</td>
<td>14/23</td>
</tr>
<tr>
<td>(30%)</td>
<td>(61%)</td>
<td></td>
</tr>
</tbody>
</table>

Three-day-old insects were given an infectious blood meal (6.0-7.0 log<sub>10</sub> TCID<sub>50</sub>/ml) spiked with the appropriate AHSV strain and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 1.75 log<sub>10</sub> TCID<sub>50</sub>/fly).

¹ Values rounded up or down to nearest integer

**DI rate:** Disseminated-infection rate

Figure 4-2: Infection rates of two reassortant viruses and their parent strains in two colonies of *C. sonorensis* – Experiment I (1999)

Three-day-old insects were given an infectious blood meal spiked with the appropriate AHSV strain (6.0-7.0 log<sub>10</sub> TCID<sub>50</sub>/ml) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 1.75 log<sub>10</sub> TCID<sub>50</sub>/fly).
Experiment II

The results of the second experiment, carried out two years after experiment I, are shown in Table 4-5. Figure 4-3 (page 95) illustrates that the VC colony is highly susceptible to infection with AHSV-8wt and AHSV-A79 but much less so to infection with AHSV-3att or AHSV-A790. There is a significant difference in IRs achieved by AHSV-A79 and AHSV-A790 in this colony (AHSV-A79>AHSV-A790; $\chi^2=49.726$, $p<0.001$, $d.f.=1$). The NVC colony recorded lower IRs, which are fairly uniform for the four virus strains tested: There was no significant difference between the two reassortant viruses ($\chi^2=1.703$, $p=0.192$, $d.f.=1$). The only statistically significant differences (although not highly so) in the NVC colony were for the virus pairs AHSV-8wt - AHSV-A790 ($\chi^2=9.590$, $p=0.002$, $d.f.=1$) and AHSV-8wt - AHSV-3att ($\chi^2=5.620$, $p=0.018$, $d.f.=1$). IRs were significantly higher with the VC than with the NVC colony for all four viruses (AHSV-3att: $\chi^2=10.138$, $p=0.001$, $d.f.=1$; AHSV-A790: $\chi^2=12.394$, $p<0.001$, $d.f.=1$; AHSV-A79: $\chi^2=86.825$, $p<0.001$, $d.f.=1$; AHSV-8wt: $\chi^2=56.169$, $p<0.001$, $d.f.=1$).

Table 4-5: Infection prevalences of two reassortant viruses and their parent strains in two colonies of C. sonorensis – Experiment II (2001)

<table>
<thead>
<tr>
<th></th>
<th>Vector-competent (VC) colony</th>
<th>Non-competent (NVC) colony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AHSV-3att</td>
<td>AHSV-A790</td>
</tr>
<tr>
<td>Number positive/number tested</td>
<td>26/73 (36%)</td>
<td>29/91 (32%)</td>
</tr>
<tr>
<td>Number with disseminated infection/number tested (DI rate in percent)$^1$</td>
<td>1/73 (1%)</td>
<td>2/91 (2%)</td>
</tr>
<tr>
<td>Number with disseminated infection/number positive (percent)$^1$</td>
<td>1/26 (4%)</td>
<td>2/29 (7%)</td>
</tr>
</tbody>
</table>

Three-day-old insects were given an infectious blood meal spiked with the appropriate AHSV strain (5.75-6.5 log$_{10}$TCID$_{50}$/ml) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log$_{10}$TCID$_{50}$/fly).

$^1$ Values rounded up or down to nearest integer

DI rate: Disseminated-infection rate
Figure 4-3: Infection rates of two reassortant viruses and their parent strains in two colonies of *C. sonorensis* – Experiment II (2001)

Three-day-old insects were given an infectious blood meal spiked with the appropriate AHSV strain (5.75-6.5 log<sub>10</sub> TCID<sub>50</sub>/ml) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log<sub>10</sub> TCID<sub>50</sub>/fly).

Figure 4-4: Proportion of insects with a fully disseminated infection (DI rates) in two colonies of *C. sonorensis* – Experiment II (2001)

Three-day-old insects were given an infectious blood meal spiked with the appropriate AHSV strain (5.75-6.5 log<sub>10</sub> TCID<sub>50</sub>/ml) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log<sub>10</sub> TCID<sub>50</sub>/fly).
Figure 4-4 (page 95) shows DI rates for experiment II (also see Table 4-5, page 94). It illustrates a statistically significant difference in DI rates between the VC and NVC colonies for AHSV-8wt (VC>NVC; $\chi^2=44.121$, $p<0.001$, $d.f.=1$) and AHSV-A79 (VC>NVC; $\chi^2=63.602$, $p<0.001$, $d.f.=1$), but not for AHSV-A790 (VC>NVC; $\chi^2=0.093$, $p=0.760$, $d.f.=1$) and AHSV-3att (NVC>VC; $\chi^2=0.920$, $p=0.338$, $d.f.=1$). There is also a statistically significant difference between AHSV-A79 and AHSV-A790 in the VC colony ($\chi^2=84.506$, $p<0.001$, $d.f.=1$) and in the NVC colony ($\chi^2=11.681$, $p=0.001$, $d.f.=1$), with the DI rate being the higher for AHSV-A79 in both cases.

![Figure 4-5: Number of insects with a fully disseminated infection as a proportion of infected insects from the two colonies of *C. sonorensis* – Experiment II (2001)](image)

Three-day-old insects were given an infectious blood meal spiked with the appropriate AHSV strain ($5.75-6.5 \log_{10} \text{TCID}_{50}/\text{ml}$) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: $0.75 \log_{10} \text{TCID}_{50}/\text{fly}$).

Figure 4-5 illustrates the proportion of infected insects that developed a fully disseminated infection. There is a significant difference between AHSV-A79 and AHSV-A790 in the VC colony ($\chi^2=47.077$, $p<0.001$, $d.f.=1$) and in the NVC colony ($\chi^2=11.849$, $p=0.001$, $d.f.=1$), with higher values for AHSV-A79 in both cases. The differences between the VC and the NVC colonies are insignificant (AHSV-A79:
\[ \chi^2 = 0.757, p = 0.384, \text{d.f.} = 1; \text{AHSV-A790: } \chi^2 = 0.496, p = 0.481, \text{d.f.} = 1 \] or on the verge of statistical insignificance (AHSV-8wt: \[ \chi^2 = 4.237, p = 0.040, \text{d.f.} = 1; \text{AHSV-3att: } \chi^2 = 4.226, p = 0.040, \text{d.f.} = 1 \]). It is noteworthy that for AHSV-3att and AHSV-A790, the proportion of insects with a fully disseminated infection is higher with the NVC than with the VC colony and, for AHSV-3att, significantly so.

### 4.3.4 Susceptibility of C. sonorensis to AHSV-6wt

The results in Table 4-6 show that *C. sonorensis* insects do not develop detectable virus titres when AHSV-6wt is imbibed. The results also show that AHSV-6wt replicates in *C. sonorensis* to high titres (\( \geq 3.5 \log_{10} \text{TCID}_{50}/\text{fly} \)) following intrathoracic inoculation (as described in section 2.3.4, page 58).

<table>
<thead>
<tr>
<th>Table 4-6: Infection rates of AHSV-6wt in <em>C. sonorensis</em> (VC colony)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infectious blood meal</strong></td>
</tr>
<tr>
<td>9 dpi</td>
</tr>
<tr>
<td>Infection rate (%)</td>
</tr>
<tr>
<td>Range (log_{10}TCID_{50}/fly)</td>
</tr>
</tbody>
</table>

Three-day-old insects were given an infectious blood meal spiked with AHSV-6wt (6.5 log_{10}TCID_{50}/ml) and incubated for 9 days at 25°C. They were then stored at -70°C and individually assayed for AHSV (minimum detection level: 0.75 log_{10}TCID_{50}/fly).

### 4.4 Discussion

**AHSV titre that indicates a fully disseminated infection in C. sonorensis**

The virus infectivity assay of insect heads shows that the titre that indicates a fully disseminated infection of *C. sonorensis* with AHSV lies within the range 2.5 to 3.0 log_{10}TCID_{50}/insect. Within this range the vast majority of insects had a fully disseminated infection, although a small number (two individuals) showed evidence of a localised infection. A control experiment shows that the orally refractory *C. nubeculosus* does not contain detectable virus titres at 9 dpi, indicating that AHSV cannot survive in *Culicoides* for this period without replication. This is further
supported by the absence of AHSV-9 from *C. nubeculosus* at 4 dpi (Wellby et al., 1996). In addition, there was a complete and consistent absence of detectable AHSV-6wt from the orally infected, susceptible (VC) *C. sonorensis* colony at 9 dpi (Table 4-6, page 97). The presence of infectious AHSV in orally infected *C. sonorensis* at 9 dpi must therefore be due to virus replication in the insect.

The experiment for the detection of AHSV-transmission across a membrane failed due to the very poor feeding rates. *Culicoides* midges are well known for being reluctant blood-feeders when kept in isolation. In future experiments, it might be possible to circumvent this problem by membrane-feeding one marked, infected female in the presence of circa 20 uninfected *Culicoides*. No logical explanation could be found for the regular failure of insects to salivate upon Malathion application. Increased concentrations of Malathion did not result in salivation but induced rapid death of the majority of insects.

In combination, the data in section 4.3.1 (page 88) strongly suggest that virus titres below $2.5 \log_{10} \text{TCID}_{50}$ arise from a localised, not fully disseminated infection of the insect. The presence of virus in all insect heads associated with bodies containing $\geq 3.0 \log_{10} \text{TCID}_{50}$ demonstrates that this virus titre always indicates a fully disseminated infection of *C. sonorensis* with AHSV. The inconsistent association of $2.5$ and $2.75 \log_{10} \text{TCID}_{50}$ titres with virus detection in the insect head may either reflect the limitations of the virus assay or variation between individual insects, or both. The virus infectivity assay used for this work is generally considered to possess an accuracy of $\pm 0.5 \log_{10} \text{TCID}_{50}$, which means that all the variation in the data can easily be explained within the inherent variability of the test. Consequently, for the purpose of this work, a titre of $2.5 \log_{10} \text{TCID}_{50}$/fly or above was chosen as an indicator of a fully disseminated infection in the insect.

The results show that the virus titre that indicates a fully disseminated infection of *C. sonorensis* with AHSV is similar to that indicative of a fully disseminated BTV infection, i.e. $2.5$ or $3.0 \log_{10} \text{TCID}_{50}$/fly (Jennings & Mellor, 1987; Fu, 1996). This is an important reference point for vector competence studies in the *C. sonorensis*-AHSV model since it distinguishes between potentially transmissive and non-transmissive vector insects. With this knowledge the epidemiological significance of laboratory-based susceptibility studies can be assessed. The fact that the titres indicative of a disseminated AHSV and BTV infection are both within the region from $2.5$ to $3.0$
log₁₀TCID₅₀/insect suggests that similar figures might apply to other orbiviruses in C. sonorensis. Therefore, vector competence studies with, for instance, EHDV, might also benefit from this information. The data confirm previous experiments showing that C. sonorensis is a competent laboratory vector of AHSV (Mellor et al., 1975) and strongly suggest that field populations of this North American species could replicate and spread AHSV if this virus were to be introduced into areas where the vector occurs. C. sonorensis is widespread and often abundant in the south and west of the USA, where it transmits BTV (Figure 1-1, page 13).

Effect of insect age on the susceptibility to oral infection with AHSV

This experiment provides evidence that teneral adults (less than three days old) are more likely to develop a fully disseminated infection with at least one AHSV strain than older adults. Although the experiment was carried out only once and with limited numbers, the difference between the two and three day old insects is statistically highly significant. The most obvious explanation for the higher susceptibility of teneral adults to virus dissemination is that an immature adult’s midgut is likely to be considerably more “leaky” than in older insects. This view is supported by the observation of a high proportion of “just emerging” insects in the two day old batch used in this experiment. While proof of the higher incidence of a “leaky gut” in teneral adults would require extensive electron microscopy, the oral infection of two-day-old C. sonorensis with AHSV-6wt could also help to resolve this question. The identification of AHSV-6wt-positive insects at 9 dpi would strongly suggest that a “leaky gut” is more common at two days of age than in older insects since this virus failed to infect three-day-old insects by the oral route but replicated following IT inoculation.

The epidemiological significance of increased oral infection rates in teneral insects is doubtful. Feeding rates of the two-day-old insects were very poor in the laboratory. In a natural environment, insects might not seek a blood meal at all at this age. However, the finding is very important to vector competence studies with laboratory colonies since it emphasises the importance to always compare insects of the same age.
Vector competence of the VC and NVC colonies of C. sonorensis

The disseminated-infection (DI) rates, as an indicator of vector competence, show that the previous selection for and against vector competence for AHSV-9 have lead to similar changes in vector competence for some, but not all AHSV strains used in this work (experiments I and II): AHSV-8wt and AHSV-A79 achieve consistently and significantly higher DI rates in the VC colony than in the NVC colony, while AHSV-3att and AHSV-A790 either show no significant differences between the two insect colonies or (in the case of AHSV-A790 in experiment I) the difference between VC and NVC colony verges on being statistically insignificant. The high reproducibility of the results illustrates that the level of susceptibility to AHSV is a stable trait in the VC and NVC colonies. At a generation interval of three to four weeks, the time span of about two years between the initial and the repeat experiments undertaken in the present work equals approximately 25-35 insect generations, while the later experiments were carried out about six years after the original selection for vector competence was completed. This means that the VC and NVC colonies have retained significantly different susceptibility rates to AHSV for approximately 75-100 generations.

It is notable that the difference in vector competence between colonies only affects those viruses which, like AHSV-9 in the original selection process, possess the potential for achieving high infection rates in C. sonorensis (i.e. AHSV-A79 and AHSV-8wt). This pattern suggests that infection rates are a function of both virus and vector characteristics, and that varying virus characteristics alone, such as variation in segment 10, only have a notable effect on infection rates in highly susceptible insect vectors. The result is in keeping with the suggestion that C. sonorensis populations possess intrinsic susceptibility rates and that virus characteristics define how much the actual DI rate approaches the maximum DI rate = susceptibility rate. However, the presented results do not clarify whether the susceptibility rate is indeed a constant, insect defined characteristic or whether it can be indefinitely increased by increasing virus titres in infectious blood meals, possibly approaching 100% in the extreme. It is likely that with high virus titres, as with other factors (e.g. high temperature, simultaneous filarial and viral infection), the “leaky gut” may account for infection rates that actually exceed the genetically determined susceptibility rate.

The almost complete absence of significant variation in infection rates and disseminated-infection rates between the two reassortants in the NVC colony (with the
exception of the disseminated-infection rate in experiment II), coupled with highly significant differences in the VC colony, suggests that the insect host susceptibility to infection could actually relate to some host interaction with NS3/3a. However, this remains speculation.

The data for dissemination of infection in those insects that already have a detectable midgut infection represent the virus’s ability to overcome the MEB. The VC and NVC colonies exhibit little or no statistical significance in this respect in experiment II (illustrated in Figure 4-5, page 96) but this is not the case in experiment I (Table 4-4, page 93). However, as experiment II was 1 log_{10} TCID_{50}/fly more sensitive than experiment I, it was considered to provide the more accurate results. Since the change in test sensitivity from 1.75 to 0.75 log_{10} TCID_{50}/insect affects the number of insects that register with a localised midgut infection, but not the number with a fully disseminated infection, it also changes the ratio: disseminated/disseminated+localised infections.

It can be concluded that the selection process for and against vector competence in the VC and NVC colonies resulted primarily and perhaps entirely in modification of the events preceding virus dissemination from the midgut (i.e. infection of the midgut cells, replication in these cells and infection of further midgut cells). To try and elucidate whether the resistance to oral infection of some insects is due to a barrier preventing the virus from entering the midgut cells or to an inherent incompetence of these cells to replicate AHSV, attempts were made to infect insect midguts from both C. sonorensis colonies from the abluminal side (see chapter 6). This approach also tests the hypothesis that the abluminal midgut cell membranes do not differ significantly between the NVC and VC colony, which is implied in the assertion that the two colonies possess essentially the same MEB.

**Effect of variation in AHSV genome segment 10 on virus dissemination in C. sonorensis**

The work presented here shows that AHSV segment 10 has a significant effect on infection and vector status of the model vector C. sonorensis. After previous findings that genome segment 10 modulates virulence in a mammalian model (O'Hara, 1994; O'Hara et al., 1998), this is further evidence that this segment is a key player in defining virus characteristics like virulence and transmission. It is nevertheless surprising to see that variation in virus genome segment 10 (between AHSV-790 and AHSV-79 in the
VC colony) affects total detectable infection rates, both in experiment I (illustrated in Figure 4-2, page 93) and experiment II (Figure 4-3, page 95). It is surprising because NS3/3a, as a non-structural protein, cannot participate in the initiation of infection. It's involvement in virus exit from the cell led to speculation that if this protein has an effect on the outcome of infection in the insect vector, it would exert its effect by affecting the MEB, not the MIB. The virus assay's sensitivity (1.75 log_{10} TCID_{50}/fly in experiment I and 0.75 log_{10} TCID_{50}/fly in experiment II) is well below the titre indicative of a disseminated infection (2.5 log_{10} TCID_{50}/fly). This means that the number of virus-positive insects includes a varying proportion of insects with a localised midgut infection, making the total infection rates largely a measure of the virus' ability to overcome the MIB. There appears to be only one logical explanation for the resulting question of how a virus protein that is involved in cell exit apparently affects an infection barrier:

In the first stage of the infection process, only a few midgut cells become infected and consequently, virus titres remain below the current detection level of 0.75 log_{10} TCID_{50}/fly. Only the spread of progeny virus particles from the initially infected cells to other midgut cells achieves detectable virus titres and this event must be controlled by NS3/3a and occur less commonly in AHSV-A790 than in AHSV-A79. The virus spread from midgut to midgut cells could occur directly via the cell membrane dividing the two cells, via the gut lumen or even via the haemocoel. Progeny virions could bud from infected midgut cells directly into neighbouring cells. This has previously been observed in cells infected with a cypovirus (Belloncik, 1996; Belloncik et al., 1996). BTV superinfection of insect cells has been shown to take place in tissue culture (Hyatt et al., 1989) and in a similar process, progeny AHSV particles could be released into the gut lumen to then re-enter infected cells but also to enter previously uninfected midgut cells. It is possible that an analogous process takes place on the abluminal side of the midgut cell, without necessarily and immediately leading to virus dissemination through the insect body. Such a low level of virus release into the haemocoel in the absence of dissemination has been described for BTV in C. sonorensis (Fu et al., 1999). Since the insect gut has enormous potential for expansion to allow large blood meals to be ingested, the gut cell membranes fold heavily when not stretched by a recent blood meal. This might allow virus particles to emerge from the abluminal side and immediately attach to the abluminal membrane of the same or a
neighbouring midgut cell, without being disseminated across the whole insect body via the haemocoel. At this stage, the fat body might also play a role in restricting infection to the midgut (Fu et al., 1999).

Evidence for replication of AHSV below detection level has been found before in the VC colony of *C. sonorensis* (Wellby et al., 1996). It was shown that virogenesis can take place at undetectable levels during low environmental temperatures but increases to detectable levels when insects are subsequently incubated at higher temperatures. The gradual colonisation of the midgut by the virus as proposed here has previously been observed in *Culex* mosquitoes orally infected with the flavivirus Japanese encephalitis virus (Doi et al., 1967). These authors initially observed a small scale infection of the posterior midgut, followed by virus spread through the posterior midgut and then infection of the fat body. In a fourth and final step, the salivary glands and other organs became infected. In the same virus-vector system, infection of the midgut following intrathoracic inoculation was observed, suggesting that the abluminal midgut cell membrane contained suitable virus receptors (Doi, 1970). The question whether the abluminal midgut cell membrane of *C. sonorensis* can be penetrated by AHSV will be explored in chapter 6.

It is also of interest to note that in the *C. sonorensis*-BTV system, two types of midgut infection have been observed following oral infection. In the first type, virus was present in the whole cytoplasm while in the second type, virus appeared compartmentalised and was restricted to endosome-like structures within the cytoplasm (Fu, 1996). It is quite possible that these two distinct patterns reflect the varying ability of a virus strain to escape from an infected midgut cell.

Since the total infection rates discussed above represent not only insects with a fully disseminated infection but also include a significant number of insects with a localised midgut infection, it is difficult to elucidate the mechanisms responsible for different infection rates elicited by different viruses. These results are also very difficult to interpret with regard to their epidemiological significance. Since only insects with a fully disseminated infection can transmit the virus, the proportion of insects out of the total population that are able to achieve such an infection is crucial to the epidemiology of the disease. For experiment II, this DI rate is illustrated in Figure 4-4, page 95. Due to the lower sensitivity of the virus assay and the resulting quality of the data, the DI rates in experiment I were not further illustrated. However, they are included in Table
The results illustrated in Figure 4-4 show that in the VC colony, the identity of virus genome segment 10 has a massive effect on the percentage of the insect population that develop a disseminated infection. This effect is also seen in experiment I and suggests that variation in this AHSV genome segment is of major significance to the epidemiology of the disease. The presence of various different types of this segment (α, β, γ; see section 1.2.3, page 26) seems to indicate that they represent adaptations to distinct environmental conditions, vector species or even to the interaction of NS3/3α with other viral proteins. To establish that variation in segment 10 can affect infection rates not only in the laboratory, but also in field vectors of AHSV, both reassortants were used for vector competence studies with wild-caught C. imicola and C. bolitinos (chapter 5).

While the proportion of insects with a disseminated infection is a good indication of potential transmission in the field, it does not allow a judgement to be made on whether it is the MIB or the MEB that leads to the observed differences in virus dissemination. The reason for this is that on its way from the midgut to the salivary glands, the virus needs to overcome both barriers, making the disseminated-infection rate a function of both, virus competence to overcome the MIB and competence to overcome the MEB. In order to decide which part of the overall result is an effect of the MEB, it is useful to look at the proportion of insects with a detectable infection (as shown in Figure 4-3, page 93) that go on to develop a fully disseminated infection. For experiment II, these data are illustrated in Figure 4-5 (page 96) and suggest that, for both insect colonies, segment 10 has a highly significant effect on virus ability to overcome the MEB. Although this assertion is not supported by the results from experiment I, this may well be the result of its lower test sensitivity. As before, it seemed logical to consider the results of the more sensitive experiment II the more accurate of the two.

The implication of this result is that variation in NS3/3α affects virus spread within the midgut in the same way as it affects virus dissemination from the midgut through the rest of the insect body. This is not surprising since both events require a functioning cell escape mechanism. Furthermore, a heavily infected midgut will almost certainly increase the chance of sufficient progeny virus escaping from the abluminal side to cause dissemination of infection to secondary target organs. The gradual
infection of the midgut could thus be seen as a preselection process for virus strains capable of further dissemination.

*Effect of variation in other AHSV genome segments on virus infection and dissemination in C. sonorensis*

The absence of significant differences in infection rates between AHSV-8wt and AHSV-A79 suggests that variation in segments 7 (coding for the serogroup defining VP7) and 9 (coding for the core associated protein VP6), either does not dramatically affect infection rates, or the effects of these two proteins cancel each other out. VP7 has been shown to possess a binding site for *C. sonorensis* in BTV (Mertens *et al.*, 1996; Xu *et al.*, 1997; Hutchinson, 1999). However, recent sequence analysis of BTV VP7 found no evidence for significant variation in the putative receptor binding site in this protein across isolates from different continents, which are associated with different insect vectors (Wilson *et al.*, 2000). This suggests that the highly conserved VP7 is not a major factor in the evolution of mutual vector-virus preferences. The high variability of VP2, the other virus protein involved in cell attachment, makes this protein more likely to be involved in adaptation to various insect vectors. It would not be surprising if VP2 and NS3/3a, the two most variable proteins in AHSV and involved in cell entrance and cell exit, turned out to be the two major virus proteins governing virus adaptation to insect vectors. One recent study examined the effect of alternating passages of BTV in the insect vector *C. sonorensis* and the mammalian hosts sheep and cattle (Bonneau *et al.*, 2001). Although the virus was passaged only twice through the insect and once through each mammalian host, some mutations in segment two (VP2) and segment 10 (NS3) were identified and the existence of BTV as a quasispecies was confirmed. This indicates a potential for rapid evolution and almost certainly applies to all orbiviruses. Most significantly, in the same experiment it was found that *C. sonorensis* “fixed” a mutation of NS3 that occurred in the sheep by replicating this virus strain (founder effect). The present work was not designed to investigate variation in genome segment 2 but the lack of significant variation in infection rates and DI rates between AHSV-3att and AHSV-A790, which differ in segments 1 to 6 and 8, does not provide evidence for a strong effect of the variations in these genome segments, including segment 2.
Susceptibility of C. sonorensis to AHSV-6wt

The fact that AHSV-6wt does not infect C. sonorensis by the oral route but does replicate if the midgut is bypassed indicates that the failure of AHSV-6wt to replicate after oral ingestion is either due to the inability to enter the midgut cells (from the gut lumen), or to replicate in them to detectable titres (or a combination of both). This inability to overcome the MIB makes this virus strain a potentially very useful tool for the further investigation of the MIB. For instance, the production of reassortant virus strains with another parent that, like AHSV-8wt, achieves high infection rates in C. sonorensis, should allow the identification of the responsible genome segment(s). AHSV-6wt and the appropriate reassortants could be used to study the mechanisms involved, for instance using immunofluorescence or immunogold labelling techniques. An advantage of that hypothetical system over the reassortant pair A79/A790 used here would be that, since oral infection rates with AHSV-6wt are zero, it should be possible to obtain qualitative rather than quantitative data.

In the present study, AHSV-6wt was also used to attempt infection of the midgut from the abluminal side, thus hoping to establish whether the C. sonorensis midgut cells are inherently unable to replicate the virus or whether, during oral infection, the virus simply fails to enter them from the luminal side (chapter 6). This virus was also used for vector competence studies with the two South African field vectors C. imicola and C. bolitinos (chapter 5). Taken together these experiments may provide some evidence of whether the zero infection rates recorded via the oral route in C. sonorensis are the result of virus mutations during mouse brain or tissue culture passaging and extend to other Culicoides species, or whether AHSV-6wt is able to orally infect other Culicoides vector species.
5 Culicoides imicola and C. bolitinos vector competence for AHSV

5.1 Introduction and rational

The Afro-Asian C. imicola is the main field vector of AHSV and its sibling species C. bolitinos has been connected with recent outbreaks of AHS (Meiswinkel & Paweska, 1999). Both species have now also been implicated in the transmission of BTV and C. imicola is the main Old World vector of this virus (Mellor & Boorman, 1995). C. imicola has also been associated with equine encephalosis virus (Venter et al., 1999).

Neither C. imicola nor C. bolitinos have been successfully colonised. AHSV and BTV vector competence studies have therefore often been carried out using laboratory colonies of C. sonorensis as a model (Boorman et al., 1975; Wellby et al., 1996). C. sonorensis is the major North American vector of BTV (Jones et al., 1981) and a laboratory vector of AHSV (Boorman et al., 1975; Mellor et al., 1975; Wellby et al., 1996). It does not occur in the Old World and belongs to a different subgenus (Monoculicoides) from C. imicola and C. bolitinos (Avaritia). While the defined age, geno- and phenotype of the model species C. sonorensis has facilitated highly controlled experiments, it has not been possible to relate the detailed findings with this species to the situation in the Old World since the vectors are so different. The chance to carry out vector competence studies on wild-caught C. imicola and C. bolitinos at the Onderstepoort Veterinary Institute (OVI) in South Africa has therefore provided a valuable opportunity to compare the findings with C. sonorensis, reported in the previous chapter, with those obtained using field vector populations. Some of the aims of this chapter are, therefore, closely related to the aims and results of chapter 4.

The first aim of this chapter was to establish the virus titre that indicates the presence of a fully disseminated AHSV infection in C. imicola. Knowledge of this titre allows differentiation between infected but non-transmissive insects and those that are transmissive and, therefore, determine the epidemiology of the disease. The experiments carried out in chapter 4 established that adults of C. sonorensis, which develop a virus titre in excess of 2.5-3.0 log_{10} TCID_{50}/insect after oral infection, possess a fully disseminated infection and are able to transmit virus. However, no data exist for any vector species outside the C. sonorensis-AHSV/BTV model vector system.
The second aim of the work described in this chapter was to determine whether variation in AHSV genome segment 10 (and NS3/3a) can influence infection rates and possibly the vector status of the two field vectors *C. imicola* and *C. bolitinos*. Chapter 4 shows that in the model vector *C. sonorensis*, variation in segment 10 can affect virus dissemination through the insect body and consequently vector competence. These experiments were carried out using two reassortant viruses, AHSV-A790 and AHSV-A79, which differ in segment 10 only. An effect of AHSV genome segment 10 on virus dissemination in *C. imicola* and *C. bolitinos* would indicate that it does have an effect on field vector competence and disease epidemiology.

The third aim of this chapter was to determine the ability of AHSV-6wt to infect *C. imicola* and *C. bolitinos*. AHSV-6wt has previously been shown to be incapable of infecting the model vector *C. sonorensis* by the oral route (section 4.3.4, page 97). It does, however, grow to high titres following intrathoracic inoculation and is therefore capable of replicating in cells of this insect species (section 4.3.4). Failure of AHSV-6wt to infect *C. imicola* and *C. bolitinos* would suggest either that this virus strain had mutated during mouse-brain and tissue-culture passaging (passage history, see Table 2-1, page 50), or that it is adapted to another, so far unidentified, vector species.

The fourth aim of the work described in this chapter was to determine the ability of attenuated virus strains to infect the field vectors of AHSV. This might provide some indication of the ability of AHSV vaccine strains to infect field vectors and, therefore, their potential for reversion to virulence and reassortment with other virus strains in the field. These questions are of great practical significance. Vaccination is an important tool in the control of AHS and also of BT, which is caused by the orbivirus type species, BTV. Live attenuated virus vaccines have been used during the recent outbreak of AHS in Iberia (1987-1990) and the ongoing outbreak of BT in Turkey and Bulgaria (Dr. P. Mellor, personal communication).

To achieve the aims set out above, *Culicoides* insects were collected from the wild at two sites in South Africa. One of these sites was at the OVI, the other one was near Clarens in the eastern Free State (for exact locations and altitudes, see Table 2-3, page 55). The OVI was originally founded at its present location near Pretoria because of the high incidence of AHS in the area and the institute grounds still provide a reliable site for catching large numbers of *C. imicola*. *C. bolitinos*, however, is not very abundant at this location. When *C. bolitinos* was first associated with AHS outbreaks,
vector competence studies required that a suitable location for catching large numbers of this species be found. Such a location was discovered near Clarens at the South Africa-Lesotho border (Venter et al., 1998; Meiswinkel & Paweska, 1999).

In brief, insects were caught nightly with light traps and maintained as described in section 2.3.1 (page 54). They were orally infected with one of AHSV-A790, AHSV-A79, AHSV-3wt, AHSV-3att, AHSV-8wt, AHSV-8att, AHSV-6wt or AHSV-6att and incubated for 10 days as described in section 2.3.3 (page 58). Insects were then immobilised and identified to species level, placed individually in Eppendorf tubes and shipped to the UK while frozen at -70°C. Virus assays were carried out at IAH Pirbright as described in sections 2.3.5 and 2.2.4 (pages 59 and 52) and infection rates calculated for comparisons between the different insect batches.

Virus infectivity assays were successfully used to detect AHSV in the heads of C. sonorensis, demonstrating fully disseminated infections (section 4.2, page 86). The same technique was therefore attempted with C. imicola, using a batch of insects that had been infected with a AHSV-A79. This virus strain was selected after experimental determination of oral infection rates for all batches of insects showed that it generated a high infection rate.

C. bolitinos is the dominant Culicoides species at Clarens and catches of C. imicola at this location are usually insufficient to allow vector competence studies with this species (Meiswinkel, 1997). In the year of this study (2000) however, unusually large numbers of C. imicola were trapped at Clarens and became available for this work. Large numbers of C. imicola and C. bolitinos were caught together and for the first time, vector competence studies became possible with mixed batches of both insect species. Batches of the two species (and some other Culicoides species) were infected and maintained together and species identification was carried out at the end of the EIP. As a result, the vector competence of the two species could be meaningfully compared. Equally importantly, trapping of large numbers of C. imicola at OVI and Clarens, approximately 350 km apart, meant that for the first time, two spatially separated populations of C. imicola could have their vector competence levels assessed in the same year, using the same virus strains and titres, and employing exactly the same laboratory techniques.
Targeted insect trapping resulted in mixed catches of mainly but not exclusively *Culicoides* species. Typically, over 90% of insects were identified as either *C. imicola* or *C. bolitinos*. Small proportions of other *Culicoides* species were often included in the catch and were exposed to the same blood-feeding and incubation procedure as *C. imicola* and *C. bolitinos*. They were identified to species level at the end of the incubation period and assayed in the same way as *C. imicola* and *C. bolitinos* as a screen for other potential AHSV vectors.

5.2 Results

5.2.1 Oral infection of *C. imicola* and *C. bolitinos* with eight AHSV strains

Oral infection rates for batches of *C. imicola* (Onderstepoort), *C. imicola* (Clarens) and *C. bolitinos* (Clarens) batches, infected with one of the reassortant, wild-type or attenuated virus strains were determined and are shown in Table 5-1. Titres of the infectious blood meals are shown in Table 5-2 (page 111).

Table 5-1: Oral infection rates of eight AHSV strains in *C. imicola* and *C. bolitinos*

<table>
<thead>
<tr>
<th>AHSV strain</th>
<th><em>C. imicola</em> (Onderstepoort)</th>
<th><em>C. imicola</em> (Clarens)</th>
<th><em>C. bolitinos</em> (Clarens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A79</td>
<td>61/167 (37%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>61/133 (46%)</td>
<td>1/98 (1%)</td>
</tr>
<tr>
<td>A790</td>
<td>4/91 (4%)</td>
<td>16/210 (8%)</td>
<td>2/176 (1%)</td>
</tr>
<tr>
<td>3wt</td>
<td>4/205 (2%)</td>
<td>4/96 (4%)</td>
<td>4/156 (3%)</td>
</tr>
<tr>
<td>3att</td>
<td>15/308 (5%)</td>
<td>7/64 (11%)</td>
<td>5/171 (3%)</td>
</tr>
<tr>
<td>8wt</td>
<td>96/231 (42%)</td>
<td>24/52 (46%)</td>
<td>10/149 (7%)</td>
</tr>
<tr>
<td>8att</td>
<td>10/220 (5%)</td>
<td>10/94 (11%)</td>
<td>2/149 (1%)</td>
</tr>
<tr>
<td>6wt</td>
<td>0/86 (0%)</td>
<td>0/53 (0%)</td>
<td>2/128 (2%)</td>
</tr>
<tr>
<td>6att</td>
<td>0/202 (0%)</td>
<td>0/133 (0%)</td>
<td>0/162 (0%)</td>
</tr>
</tbody>
</table>

<sup>1</sup>-Number infected/number assayed (infection rate in percent, rounded to the nearest integer).

Insects were caught at Onderstepoort and Clarens with UV light traps, given an infectious blood meal spiked with the appropriate AHSV strain and incubated for 10 days at 23.5±1°C. They were then identified to species level, stored at -70°C and individually assayed for AHSV. Infection rates were calculated as the percentage of insects tested in which AHSV could be detected (minimum detection level: 0.75 log<sub>10</sub>TCID<sub>50</sub>/fly).
Table 5-2: Titre of infectious blood-meals before and after feeding (log_{10} TCID_{50}/ml)

<table>
<thead>
<tr>
<th>AHVS strain</th>
<th>C. imicola (O'poort)</th>
<th>C. imicola (Clarens) and C. bolitinos [blood-fed together]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A79</td>
<td>6.75/6.25</td>
<td>7.0/6.5</td>
</tr>
<tr>
<td>A790</td>
<td>6.25/6.25</td>
<td>6.5/6.25</td>
</tr>
<tr>
<td>3wt</td>
<td>6.5/6.25</td>
<td>6.75/6.75</td>
</tr>
<tr>
<td>3att</td>
<td>6.25/6.25</td>
<td>6.75/6.5</td>
</tr>
<tr>
<td>8wt</td>
<td>6.5/6.25</td>
<td>7.0/7.0</td>
</tr>
<tr>
<td>8att</td>
<td>6.5/6.25</td>
<td>7.25/7.0</td>
</tr>
<tr>
<td>6wt</td>
<td>6.75/6.75</td>
<td>7.25/6.75</td>
</tr>
<tr>
<td>6att</td>
<td>6.5/6.5</td>
<td>7.25/6.75</td>
</tr>
</tbody>
</table>

For illustration, three figures have been derived from the results shown in Table 5-1. Figure 5-1 shows that the reassortant virus AHVS-A79 achieves significantly higher infection rates than AHVS-A790 in two different populations of C. imicola (Onderstepoort: \( \chi^2 = 32.267, p < 0.001, d.f. = 1 \); Clarens: \( \chi^2 = 68.416, p < 0.001, d.f. = 1 \)). In

Figure 5-1: Oral infection rates of the two reassortant viruses in C. imicola and C. bolitinos

Insects were caught at Onderstepoort and Clarens with UV light traps, given an infectious blood meal spiked with the appropriate AHVS strain and incubated for 10 days at 23.5±1°C. They were then identified to species level, stored at -70°C and individually assayed for AHVS. Infection rates were calculated as the percentage of insects tested in which AHVS could be detected (minimum detection level: 0.75 log_{10} TCID_{50}/fly).
contrast, there was no significant difference in infection rates between these two virus strains in *C. bolitinos* ($\chi^2=0.008, p=0.930, d.f.=1$) which were both very low compared to the *C. imicola* results.

Differences in oral infection rates between attenuated and wild-type virus strains are illustrated in Figure 5-2. AHSV-8wt infected both populations of *C. imicola*, as well as *C. bolitinos*, to significantly higher levels than AHSV-8att (*C. imicola*-O’poort: $\chi^2=85.861, p<0.001, d.f.=1$; *C. imicola*-Clarens: $\chi^2=23.639, p<0.001, d.f.=1$; *C. bolitinos*: $\chi^2=5.557, p=0.018, d.f.=1$). AHSV-8att also infected a proportion of all three insect populations and reached an infection rate of over 10% in *C. imicola* (Clarens). AHSV-3wt and AHSV-3att both infected a proportion of all three insect populations. The infection rates for the heavily attenuated AHSV-3att exceeded those for the far less attenuated AHSV-3wt in all three populations but these differences were statistically not significant (*C. imicola*-O’poort: $\chi^2=2.940, p=0.086, d.f.=1$; *C. imicola*-Clarens: $\chi^2=2.750, p=0.097, d.f.=1$; *C. bolitinos*: $\chi^2=0.039, p=0.843, d.f.=1$). AHSV-6wt did not infect any *C. imicola* but did infect 2% of *C. bolitinos*. AHSV-6att did not infect any insects at all.

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Figure 5-2: Infection rates of wild-type and attenuated AHSV strains in *C. imicola* and *C. bolitinos*

Insects were caught at Onderstepoort and Clarens with UV light traps, given an infectious blood meal spiked with the appropriate AHSV strain and incubated for 10 days at 23.5±1°C. They were then identified to species level, stored at -70°C and individually assayed for AHSV. Infection rates were calculated as the percentage of insects tested in which AHSV could be detected (minimum detection level: $0.75 \log_{10} \text{TCID}_{50}$/fly).
Figure 5-3 compares infection rates with all eight virus strains across the two C. imicola populations and also between C. imicola and C. bolitinos. The difference between the oral infection rates of C. imicola (O'poort) and C. imicola (Clarens) with AHSV-8att (5% and 11%, respectively) was only just significant ($\chi^2=4.100$, $p=0.043$, $d.f.=1$). There were no significant differences between C. imicola (O'poort) and C. imicola (Clarens) for any of the other seven virus strains.

The infection rates for C. bolitinos differed significantly from those in C. imicola for both reassortant viruses (AHSV-A79: $\chi^2=57.786$, $p<0.001$, $d.f.=1$; AHSV-A790: $\chi^2=9.051$, $p=0.003$, $d.f.=1$), for AHSV-8wt ($\chi^2=42.670$, $p<0.001$, $d.f.=1$), AHSV-8att ($\chi^2=10.610$, $p=0.001$, $d.f.=1$) and AHSV-3att ($\chi^2=6.172$, $p=0.013$, $d.f.=1$), but not for AHSV-3wt ($\chi^2=0.497$, $p=0.481$, $d.f.=1$).

Figure 5-3: Susceptibility of C. imicola and C. bolitinos to eight strains of AHSV

Insects were caught at Onderstepoort and Clarens with UV light traps, given an infectious blood meal spiked with the appropriate AHSV strain and incubated for 10 days at 23.5±1°C. They were then identified to species level, stored at -70°C and individually assayed for AHSV. Infection rates were calculated as the percentage of insects tested in which AHSV could be detected (minimum detection level: 0.75 $\log_{10}$TCID$_{50}$/fly).
5.2.2 AHSV titre that indicates dissemination of infection in *C. imicola*

AHSV-8wt and AHSV-A79 registered the highest infection rates (Table 5-1, page 110) and also the highest maximum titres (Table 5-3) in *C. imicola* and were therefore identified as the most suitable virus strains for this experiment. Due to variable insect availability, feeding rates and survival, batches of infected insects available for assay varied considerably in numbers. The only batch large enough to make insects available for this experiment was *C. imicola* (Onderstepoort) infected with AHSV-A79. Insects were orally infected (6.75 log_{10} TCID_{50}/ml), maintained, identified and stored in the same way as those insects used for determination of oral infection rates.

No AHSV could be detected in any of the 50 *C. imicola* heads assayed. Infection rates in the corresponding bodies ranged from 0.75 to 2.75 log_{10} TCID_{50}.

Table 5-3: Maximum titres of eight AHSV strains in *C. imicola* and *C. bolitinos*  
(log_{10} TCID_{50}/fly)

<table>
<thead>
<tr>
<th>AHSV strain</th>
<th><em>C. imicola</em> (Onderstepoort)</th>
<th><em>C. imicola</em> (Clarens)</th>
<th><em>C. bolitinos</em> (Clarens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A79</td>
<td>3.0</td>
<td>3.75</td>
<td>0.75</td>
</tr>
<tr>
<td>A790</td>
<td>1.5</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>3wt</td>
<td>1.5</td>
<td>1.5</td>
<td>1.25</td>
</tr>
<tr>
<td>3att</td>
<td>2.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>8wt</td>
<td>3.25</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>8att</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6wt</td>
<td>&lt;0.75</td>
<td>&lt;0.75</td>
<td>1.5</td>
</tr>
<tr>
<td>6att</td>
<td>&lt;0.75</td>
<td>&lt;0.75</td>
<td>&lt;0.75</td>
</tr>
</tbody>
</table>

Values refer to the insect batches shown in Table 5-1. The minimum detectable titre was 0.75 log_{10} TCID_{50}/fly.
Table 5-4: Oral susceptibility of various Culicoides species to AHSV

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Number AHSV-positive/number tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. enderleini</td>
<td>0/1</td>
</tr>
<tr>
<td>C. gulbenkiani</td>
<td>0/43</td>
</tr>
<tr>
<td>C. leucostictus</td>
<td>0/10</td>
</tr>
<tr>
<td>C. nivosus</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Insects were caught with UV light traps, given an infectious blood meal spiked with AHSV and incubated for 10 days at 23.5°C. They were then identified to species level, stored at -70°C and individually assayed for AHSV. The lowest detectable titre was 0.75 log_{10} TCID_{50}/fly.

5.2.3 Infection rates of other Culicoides species

Small numbers of various other Culicoides species, trapped in the collections aimed at C. imicola and C. bolitinos, were blood-fed as chance dictated, with any one of the eight virus strains used in this study and were incubated together with C. imicola and C. bolitinos. Survivors were assayed for AHSV but no virus was detected in any of these insects (Table 5-4).

5.3 Discussion

AHSV titre that indicates dissemination of infection in C. imicola

Although virus titres in the insect bodies ranged from 0.75 (the lowest detection level) to 2.75 log_{10} TCID_{50}, no virus was detected in any of the corresponding insect heads. This either indicates that the titre indicative of a fully disseminated infection in C. imicola is higher than 2.75 log_{10} TCID_{50}, or that the virus infectivity assay failed to detect AHSV despite its presence in the insect head. C. sonorensis insects with an AHSV titre of 2.5 log_{10} TCID_{50} have been shown to have a fully disseminated infection (chapter 4). C. imicola is a much smaller insect than C. sonorensis and it seems unlikely that individuals of C. imicola would develop a titre above 2.5 log_{10} TCID_{50}, without the virus having disseminated through the whole insect body. It is possible that the result of
this experiment reflects a failure of the virus infectivity assay. The small size of *C. imicola* probably means that less virus, possibly below the minimum detection level of 0.5 log$_{10}$TCID$_{50}$/head (section 4.2, page 87), will be present in the head of an insect with a fully disseminated infection. The small insect size may also have a negative effect on the reliability of insect dissection and processing compared to *C. sonorensis*.

In the absence of data on disseminated virus infection of *C. imicola*, it can only be speculated that AHSV-8wt and AHSV-A79, which achieved maximum titres of 3.0 log$_{10}$TCID$_{50}$/fly or above (Table 5-3) in both *C. imicola* populations, would probably be transmitted by this species. The same applies to AHSV-8wt in *C. bolitinos* (maximum titre: 3.5 log$_{10}$TCID$_{50}$/fly). Since only a small proportion of insects developed titres above 2.0 log$_{10}$TCID$_{50}$/fly, the higher maximum titres might simply be a reflection of the higher infection rates elicited by the two virus strains, with an associated higher chance of detecting a number of insects with high titres. The titre of 3.0 log$_{10}$TCID$_{50}$/fly is well above that indicating a disseminated infection in the much larger *C. sonorensis* (2.5 log$_{10}$TCID$_{50}$/fly). However, *C. imicola* and *C. bolitinos* are both members of the subgenus *Avaritia*, whereas *C. sonorensis* belongs to the subgenus *Monoculicoides*. This relationship means that the latter species is more closely related for instance to the refractory palaearctic *C. [monoculicoides] nubeculosus* than to the two field vector species, illustrating the fact that *Culicoides* are a biologically highly diverse genus and that extrapolation from one species to others is fraught with uncertainty.

*Effect of variation in AHSV genome segment 10 on oral infection of C. imicola*

The significant difference in oral infection rates of *C. imicola* with AHSV-A790 and AHSV-A79 shows that variation in AHSV genome segment 10 modifies infection rates in its main field vector (Figure 5-1, page 111). The similarity of the results for two populations of *C. imicola* underlines the significance of the results and they are further supported by the similar results in the model vector *C. sonorensis* (chapter 4).

The more than five-fold increase in infection rates with AHSV-A79 over AHSV-A790 strongly suggests that the variation in genome segment 10 between different AHSV isolates is an effective way of virus adaptation to different vector species. In such a scenario, one might expect one variant segment 10 to confer an advantage over the other variant (in the form of higher oral infection rates) in some insect species, with a reverse situation in other vector species. This has not been the case in this study.
AHSV-A79 recorded higher infection rates than AHSV-A790 in *C. imicola* and *C. sonorensis* and an equal infection rate in *C. bolitinos* while AHSV-A790 never recorded higher infection rates than AHSV-A79). However, the segment 10 present in AHHSV-A790 might confer an advantage over the segment 10 in AHHSV-A79 in as yet unidentified vector species. Hypothetically, it is also possible that the competitive advantage in the insect vector of one variant segment 10 is offset by a longer viraemic period in the mammalian host caused by another variant, thus assuring the survival of two or more existing variants of this genome segment. Such a hypothesis is supported by experiments showing that variation in AHHSV segment 10 can affect virulence in a mouse model (O'Hara et al., 1998).

The high degree of variation in ability of AHHSV-A79 and AHHSV-A790 to orally infect *C. imicola*, combined with the absence of significant variation in infection rates between the reassortant strains and the respective parent that supplied genome segment 10 (A790 - AHHSV-3att; A79 - AHHSV-8wt), follows a pattern also seen in the VC colony of *C. sonorensis* (chapter 4). The data suggest that, for the virus strains used in this work, AHHSV segment 10 may have the strongest effect of all virus genome segments on the oral infection rate in the vector insect. As discussed in section 4.4 (page 105), the lack of variation between A79 and AHHSV-8wt suggests little effect of genome segments 7 or 9, or effects that cancel each other out. The consistent absence of variation between A790 and AHHSV-3att suggests the same for segments 1-6 and 8.

**Susceptibility of C. imicola and C. bolitinos to infection with AHHSV-6wt and AHHSV-6att**

The results with AHHSV-6wt and AHHSV-6att (AHHSV-6wt infected no *C. imicola* and only two *C. bolitinos* insects, AHHSV-6att infected no insects at all) contrast with the results for all other virus strains used in this study (each of them infected both *C. imicola* and *C. bolitinos* insects). These differences are probably not due to the passage histories of AHHSV-6wt and AHHSV-6att, since these differ little from those of the wild-type and attenuated strains, respectively, of serotypes eight and three (Table 2-1, page 50). The completely negative results for AHHSV-6wt in *C. imicola* are supported by the results of *C. sonorensis*-studies with this virus, with no infected insects recorded (chapter 4). However, chapter 4 also showed that AHHSV-6wt can replicate in the insect if inoculated intrathoracically, indicating that this virus is incapable of breaching the midgut barriers in orally infected *C. sonorensis*. Similarly, the negative results with *C.
imicola may be due to an inability of AHSV-6wt to cross the midgut barriers in this species.

There is a possibility that a “leaky gut” is the cause of AHSV-6wt infection of the two positive C. bolitinos. If this is so then the apparent higher incidence of “leaky guts” in C. bolitinos, compared to C. imicola and C. sonorensis (none of these recorded an infection following oral infection with AHSV-6wt) could be due to the potentially higher larval rearing temperatures of the dung-breeding C. bolitinos. An increase in larval rearing temperature has been shown to lead to leaky guts in C. nubeculosus imagoes (Wittmann, 2000).

If infection of C. bolitinos is in fact caused by a “leaky gut” then the failure of AHSV-6wt to infect by the oral route involves one or more of these processes: Attachment to the midgut cells, entry into the midgut cells and replication in them. In short, it fails to overcome the MIB. The ability of AHSV-6wt to abluminally infect and replicate in Culicoides midgut cells is explored in chapter 6 and compared to AHSV-8wt, which successfully infects C. sonorensis, C. imicola and C. bolitinos by the oral route.

The oral infection rates registered with AHSV-6wt in the model and field vectors included in this study raise the question of how this virus strain may survive in the field. One possibility is that it uses an as yet unidentified vector. Alternatively, it might use C. imicola or C. bolitinos as a field vector, but then the population present at the time and place of the outbreak that led to the virus isolation must have differed significantly in vector competence from the populations used in this experiment.

**Susceptibility of C. imicola and C. bolitinos to AHSV-3att and AHSV-8att**

This is the first time that heavily attenuated AHSV strains have been shown to infect their field vectors. Although no other data are available for orbiviruses, concern about the potential spread of live attenuated viruses following vaccination of humans has led to more extensive research on other arthropod-borne viruses. Work carried out on attenuated strains of Rift Valley fever, Chikungunya, dengue-1 and dengue-2 viruses suggests that all these viruses can successfully infect laboratory colonies of Aedes aegypti by the oral route (Turell & Rossi, 1991; Turell & Malinoski, 1992; Khin et al., 1994; Jirakanjanakit et al., 1999). However, no evidence has been published to show that heavily attenuated arboviruses can infect field vectors, collected from the wild in
areas where the virus is endemic. Although AHSV-3att achieved marginally higher infection rates than AHSV-3wt in both *C. imicola* populations and also in *C. bolitinos*, this cannot be unequivocally interpreted as an effect of attenuation since the two isolates may not have been derived from the same parent strain. Nevertheless, the oral infection of two field vector species with AHSV-3att and AHSV-8att shows that the live attenuated AHSV vaccine strains which are currently used in commercial vaccines will also be likely to be able to infect *Culicoides* species. To assess this risk in detail, AHSV strains included in the commercial vaccines should be used in vector competence studies similar to the ones described in this chapter. Vaccine strains should also be checked for their potential to cause viraemia. There is some limited evidence to suggest that tissue-culture attenuated vaccine strains of AHSV do not induce detectable viraemia in ponies (House, 1998). Transmission studies involving the exposure of recently vaccinated horses (and other equids) to the bite of susceptible *Culicoides* vectors could verify the significance or otherwise of that finding. However, it could be dangerous to extrapolate from the possible negative outcome of such studies to suggest that the investigated vaccines are safe, since in a field situation the number of vectors and potentially viraemic horses is likely to be much greater than in simulated laboratory experiments. An AHS outbreak in non-endemic areas is likely to lead to emergency vaccination in the presence of the field virus, which would almost certainly mean that some equids would contain simultaneously the wild type and the vaccine virus strains. This could result in reassortment, with the potential to generate virus strains with new virulence and antigenic characteristics. The high oral infection rates in *Culicoides* insects with AHSV-A79 (the virus which resulted from the reassortment of the heavily attenuated AHSV-3att with the only lightly attenuated AHSV-8wt) emphasise that this is a realistic scenario. The use of non-replicative vaccines appears to be the only really safe way out of this situation.

**Comparison of oral infection rates between *C. imicola* and *C. bolitinos***

Infection rates in *C. bolitinos* differ from those in *C. imicola* in two respects. Firstly, *C. bolitinos* infection rates are lower for all virus strains except for AHSV-6wt and AHSV-6att, which have been discussed above. This does not, however, mean that *C. bolitinos* is a universally poorer vector than *C. imicola*. A previous study recorded AHSV-5 infection rates of 20.6% in *C. bolitinos*, compared to just 8.5% in *C. imicola* (Venter et al., 2000).
Secondly, in *C. bolitinos*, there is no significant difference between the infection rates recorded by AHSV-A790 and AHSV-A79, i.e. the variation in virus genome segment 10 had no obvious effect. This could indicate that neither type of segment 10 is particularly suited to infection of this vector species, or just this particular population. The results for *C. bolitinos* resemble those for the NVC colony of *C. sonorensis* at IAH Pirbright, which also combined low infection rates with lack of variation in infection rates between these reassortant viruses (chapter 4). This pattern might suggest that in highly susceptible vectors (such as the *C. sonorensis* VC colony and *C. imicola*) variation of segment 10 enables some virus strains (e.g. AHSV-A79) to achieve higher infection rates than others, while in vectors with a low oral susceptibility (such as *C. sonorensis* NVC colony and perhaps *C. bolitinos*), it confers no particular advantage or disadvantage.

The following is highly speculative but it can be argued that highly susceptible insect vectors vary from less susceptible ones by having a factor that interacts with the (segment-10 encoded) NS3/3a. This factor could be the identity or expression level of a NS3/3a-interacting host cell protein. In this case it might be possible to identify such a protein, using cellular techniques like immunoprecipitation, yeast-2-hybrid systems and GST fusion assays. The results of such experiments could be used to compare the midgut cells of *C. sonorensis* from the VC colony with those from the NVC colony since it is a factor within these cells that is the basis of the difference between the two colonies. Alternatively, if highly specific anti-NS3 antibodies could be produced, this should allow immunofluorescence or electron microscopy studies to screen for NS3-co-localisation with cellular structures and a range of cellular proteins, for which antibodies are commercially available. To identify differences between vector-competent and non-competent insects, it would be necessary to carry out these experiments *in situ*, i.e. in the midgut cells. The VC and NVC *C. sonorensis* colonies would be an ideal tool for such studies.

*Susceptibility of two *C. imicola* populations to AHSV*

This is the first time that it has been possible to determine the vector competence levels of *C. imicola*, from two different geographical locations, for an orbivirus during the same insect season. The amazingly similar pattern of infection rates for the two populations of *C. imicola*, which originated from locations 350 km apart, suggests that
insects from the two sources were genetically closely related at the time these experiments were carried out. Since *C. imicola* have been caught in locations situated between Onderstepoort and Clarens, the most rational explanation for these results is that there is an uninterrupted gene flow between these two sites. This view is supported by the recent investigation of genetic variability within the *C. imicola* species complex (Sebastiani et al., 2001). Comparing patterns of randomly generated DNA fragments in insects from various locations across Southern Africa, it was found that there was less genetic variation between geographical populations in *C. imicola* than in other members of the species complex, e.g. *C. bolitinos*. Among others, the study included *C. imicola* from Onderstepoort and from several locations more remote from Onderstepoort than Clarens. Clarens was not included since until recently it was virtually *C. imicola*-free (Meiswinkel, 1997). The authors suggested that within southern Africa, *C. imicola* might be genetically quite homogeneous, a characteristic that may be facilitated by its utilisation of moist, organically enriched soil for breeding. Ideal breeding conditions are often provided by human activity and can cover large areas, enabling *C. imicola* to spread and preventing genetic isolation. In view of the results obtained in this chapter and the genetic evidence described above, *C. imicola* from different South African locations should be considered as parts of one large population. Interestingly, a recent study of the genetic variability of *C. impunctatus*, which is as ubiquitous in the UK as *C. imicola* is in South Africa, concluded that insects from Scotland and from southern England were very closely related (Dr. S. Carpenter, personal communication).

Although the present study indicates that *C. imicola* can be quite uniform in its susceptibility to AHSV over a distance of several hundred kilometres, this might not always be the case. Depending on prevailing weather conditions, the situation may vary from season to season. Great differences in ambient temperatures between two locations, for instance, could lead to differences in vector susceptibility since larval rearing temperatures and adult incubation temperatures during the EIP have both been shown to affect oral infection rates of *Culicoides* insects with AHSV (Wittmann, 2000). The situation may also differ in regions where geographic features hinder the active and passive spread of insects. Hence, up to date evaluations of insect vector competence will always be desirable to provide an accurate estimate of the risk of disease outbreaks.
Variation of vector susceptibility to AHSV over time

Vector competence studies similar to the ones described in this chapter were carried out with *C. imicola* from Onderstepoort and *C. bolitinos* in 1999, approximately one year before the completion of the experiment in the present study (Venter et al., 2000). The virus strains designated as AHSV-3wt and AHSV-8wt here were also used in the 1999 study. Between the two sets of results, some striking similarities exist. AHSV-8(wt) reached a much higher infection rate (26.8%) than AHSV-3(wt) (0%) in *C. imicola*, just as in the present study (Table 5-1, page 110). Also, a low proportion of *C. bolitinos* proved susceptible to infection with these two viruses (AHSV-8: 1.7%, AHSV-3: 3.8% infection rate), which again bears similarity to the results obtained in the present study. These similarities are a sign of relative stability in the two insect species’ susceptibility to AHSV. However, additional to the present work, *C. bolitinos* was highly susceptible to one virus isolate (AHSV-5) with an infection rate of 20.6%. This illustrates that some populations of *C. bolitinos* can be a highly competent AHSV vector for certain strains or serotypes of virus.

Susceptibility of other Culicoides species to AHSV

The failure of AHSV to infect *C. enderleini*, *gulbenkiani*, *leucostictus* and *nivosus* by the oral route confirms a previous study with the same result for these four and an additional 11 *Culicoides* species (Venter et al., 2000). As yet, AHSV has not been recovered at the end of a 10-day EIP from any Old World, non-Avaritia species. Nevertheless, due to the limited amount of data available (Venter et al., 2000, and this study), the ability of other *Culicoides* species to become infected and transmit AHSV cannot be ruled out. Moreover, the length, temperature and humidity of extrinsic incubation selected in the present study may not suit all species. The consistent absence of AHSV from other *Culicoides* species in the present work serves as a control, confirming that the virus detected in *C. imicola* and *C. bolitinos* at the end of the 10-day EIP is not residual virus from the infectious blood meal but is replicating virus.
6 Immunofluorescence studies of AHSV replication in insect cells and Culicoides midguts

6.1 Introduction and rational

The experiments described in chapter 4 show that the ability of AHSV to overcome the MIB of the vector C. sonorensis can be controlled by an insect-defined factor, such as genetic selection in the vector. On the other hand, variation in segment 10 of AHSV, encoding the non-structural protein NS3/3a, has also been shown to affect oral infection rates in C. sonorensis (see chapter 4), which implies that this viral protein (i.e. a virus-defined factor) may influence infection. However, as a non-structural protein, NS3/3a is absent at the time of initial cell attachment and infection by virus particles. Consequently, it is difficult to understand how it can affect these processes. In this context, it is likely that the initial cycle of infection and replication in vector midgut cells is below the level of detection of the virus infectivity assays used in the present work (0.75 log_{10} TCID_{50}/insect) and that the presence of replicating virus, therefore, only becomes detectable during additional cycles of infection. This secondary spread of virus would be in the presence of NS3/3a and could be controlled by it, thereby leading to the different infection rates detected for AHSV-A79 and AHSV-A790. This suggestion is supported by the findings of chapter 4, where the ability of AHSV to overcome the MEB of C. sonorensis seems to depend on the identity of NS3/3a. However, the findings of chapter 4 did not reveal the mechanisms by which this occurs.

The overall aim of this chapter was to investigate the mechanisms by which NS3/3a affects the virus spread within the vector’s midgut cells and from the midgut cells into the haemocoel. Immunofluorescent microscopy was chosen as a potentially quick and reliable method of localising NS3/3a within infected cells. The overall aim was approached via four closely related objectives:

The first objective was to develop a reliable method for identifying infected cells in KC tissue culture and in Culicoides midguts. Efforts concentrated on guinea pig anti-VP7 polyclonal antibody because of the high conservation of VP7 within the AHSV species and the availability of the antibody in the laboratory. VP7 is highly hydrophobic and has been found, in the shape of hexagonal crystals, in BHK-21 cell lysates (Burroughs et al., 1994). It was reasoned that if VP7 produced similarly conspicuous
structures in live KC and *Culicoides* midgut cells then this might be an easy and reliable way of detecting AHSV infection. An anti-VP7 antibody has been used before for the detection of BTV in *Culicoides* insects (Fu *et al.*, 1999).

The second objective was to develop a reliable way of visualising NS3/3a in KC cells and in *Culicoides* midguts. To achieve this aim, two anti-NS3/3a antibodies were obtained from collaborating laboratories (PAb-SA and PAb-1-117, Table 6-1, page 127) and assessed in KC cell immunofluorescence studies. Polyclonal anti-NS3/3a-peptide sera were also produced at IAH Pirbright and tested in KC cells to address this issue. Anti-NS3/3a antibodies were used for Western blot analysis of infected and uninfected KC cell lysates to check for cross-reactions with other viral and cellular proteins.

The third objective was to determine whether or not AHSV replicates (to low levels, which are undetectable by the virus infectivity assay) in those *Culicoides* that test negative in virus infectivity assays following attempted oral infection. *C. nubeculosus* were used for this purpose since this is a species that has been shown to be refractory to oral infection with AHSV and BTV unless very high infecting titres are used (Mertens *et al.*, 1996) or unless the larval rearing temperatures are raised to near lethal levels (Wittmann & Baylis, 2000). Batches of *C. nubeculosus* insects were orally fed with either AHSV-6wt or AHSV-8wt. Their midguts were labelled with an anti-VP7 antibody after an EIP of 10 days and subjected to immunofluorescence microscopy. The experiment was carried out in the same way with insects from the *C. sonorensis* (VC) colony. AHSV-6wt apparently failed to orally infect *C. sonorensis* in previous experiments, (chapter 4), but it remained unclear if it did infect some insects at a very low level (i.e. titre). However, AHSV-8wt achieved high infection rates in this colony (chapter 4). Here, evidence of midgut infection should be easy to find and would serve as a positive control. To ensure that the structures detected by the anti-VP7 antibody are an indicator of infection and do not detect residual virus remaining from the infectious blood meal, further *C. nubeculosus* insects, orally infected with AHSV-8wt, were dissected and used for immunofluorescence after EIPs of 0, 1 and 2 days, respectively.

The fourth objective was to investigate the ability of *Culicoides* midgut cells to become infected from the abluminal side. *C. sonorensis* (VC) were inoculated intrathoracically (IT) with either AHSV-8wt or AHSV-6wt. Insects from the *C. sonorensis* (NVC) colony and *C. nubeculosus* were also inoculated IT with AHSV-6wt. All insects were dissected and their intestines subjected to immunofluorescence with
anti-VP7 antibody after an EIP of 10 days. *C. sonorensis* has been found to replicate AHSV-6wt following IT inoculation (chapter 4) and *C. nubeculosus* has also been found to replicate IT inoculated AHSV (Mellor *et al.*, 1975). Replication of AHSV in the midgut following IT inoculation could indicate the presence of AHSV receptors in the ab luminal midgut membrane of the affected insect batches. In the case of AHSV-6wt, it would also show that the failure of the virus to orally infect insects (chapter 4) represents a failure to enter the midgut cells from the luminal side, rather than an inability to replicate in them. In addition, infection of midgut cells from the ab luminal side would lend some credibility to the hypothesis that cell-to-cell spread within the midgut may occur via the haemocoel (chapter 4). The results obtained for the VC and the NVC colonies also test the assertion made in chapter 4 that selection of these colonies did not change the character of their ab luminal membrane, representing the MEB.

6.2 Methods

6.2.1 Production of polyclonal antibodies against a NS3/3a peptide

Using the sequence analysis program GCG 10, a potentially highly antigenic amino acid sequence, which is conserved in many variants of AHSV NS3/3a, was chosen (Figure 6-1). The amino acid sequence (ERLR DPEPI RQIKK) is located at aa 77-90 in the N-terminal region of the protein. A cysteine residue was added to the N-terminal and the peptide was produced as an amide by Dr. L. Hunt at IAH Compton. The amide structure mimics the internal peptide better than the alternative acid structure while the addition of a cysteine residue allowed the peptide to be conjugated to the carrier protein, keyhole limpet haemocyanin (KLH). KLH is the oxygen-carrying molecule of the mollusc *Diodora aspera* (Figure 6-2) and was used as a conjugate to enhance the molecular size and the antigenic properties of the molecule (Harlow & Lane, 1988). Conjugation of the peptide to KLH was carried out using the cross-linking molecule SMCC (see protocol in section 2.5.15, page 65).

Five guinea pigs and two rabbits were inoculated subcutaneously with 1 ml of KLH-conjugated peptide in PBS, containing the equivalent of 1 µg/ml peptide. The injection was repeated seven times at 14-day intervals. All animals were exsanguinated
14 days after the last booster injection. Serum was obtained by leaving the blood to clot overnight and centrifuging the supernatant at 100 g for 5 min. It was then stored at -20°C. Small aliquots for day-to-day use were stored at 4°C.

Figure 6-1: Predicted secondary structure and antigenic regions of AHSV-NS3/3a

Figure 6-2: The keyhole limpet *Diodora aspera*

(http://www.ocnms.nos.noaa.gov/LivingSanctuary/limpet.html)
6.2.2 Antibodies used in immunofluorescence studies

All primary antibodies except for the monoclonal "anti-tubulin" used in this work are polyclonal antibodies (PAbS) and listed in Table 6-1. A total of seven PAbS, named PAb-R1, -R2 and -G1 through -G5, were produced at IAH Pirbright as a part of this project. Staining was by the method detailed in section 2.5.17 (page 65). Antibodies and stains were used in various combinations as indicated in the legends of the images obtained (section 6.3). No primary antibodies were used in control samples. PAb-VP7 was used at a concentration of 1/2000 and anti-NS3/3a antibody concentrations ranged from 1/100 to 1/5000. All commercially available stains and antibodies were used at the concentrations recommended by the manufacturer.

Table 6-1: Primary antibodies used in immunofluorescence microscopy

<table>
<thead>
<tr>
<th>Designation</th>
<th>Raised in</th>
<th>Raised against</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAb-VP7</td>
<td>Guinea pig</td>
<td>Purified AHSV-9 VP7</td>
<td>Dr. P. Mertens, IAH Pirbright</td>
</tr>
<tr>
<td>PAb-SA1</td>
<td>Rabbit</td>
<td>AHSV-3 NS3/3a2</td>
<td>Dr. A. van Dijk, OVI Pretoria, South Africa</td>
</tr>
<tr>
<td>PAb-1-1171</td>
<td>Rabbit</td>
<td>AHSV-4 (Morocco) NS3 aa 1-117 (N-terminal)3</td>
<td>Dr. S. Zientara, AFSSA, Maisons Alfort, France</td>
</tr>
<tr>
<td>PAb-R1 and PAb-R21</td>
<td>Rabbit</td>
<td>ERLR DPEPI RQIKK (NS3 peptide)</td>
<td>See section 6.2.1 (page 125)</td>
</tr>
<tr>
<td>PAb-G1 to PAb-G5 (5 antibodies)1</td>
<td>Guinea pig</td>
<td>ERLR DPEPI RQIKK (NS3 peptide)</td>
<td>See section 6.2.1 (page 125)</td>
</tr>
<tr>
<td>Anti-tubulin</td>
<td>Mouse</td>
<td>α-tubulin</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

1 PAb-SA, PAb-1-117, PAb-R1, -R2, -G1 to -G5 are collectively referred to as anti-NS3/3a antibodies.
2 No further information regarding the precise virus strain or NS3/3a type was available.
3 This virus strain was also supplied by Dr. Zientara and was used together with PAb-1-117 throughout.

6.2.3 Immunofluorescence studies in C. sonorensis (KC) cells

KC cells were grown on 13 mm glass cover slips to 50% confluency and infected with one of AHSV-3att, AHSV-A790, AHSV-A79, AHSV-8wt, AHSV-6wt and AHSV-4 (approximate multiplicity of infection - MOI = 10). After 20 h incubation at 29°C, cells were fixed (section 2.5.16, page 65) and immediately stained for immunofluorescence microscopy, using one of the nine anti-NS3/3a antibodies (Table 6-1) in combination with PAb-VP7 and the DNA stain TO-PRO-3. Staining was
followed by mounting on microscopy slides. Microscopic examination was carried out immediately using a non-confocal microscope and within four days using a confocal microscope. The same procedure was applied to uninfected control cells.

6.2.4 Immunofluorescence studies in Culicoides midguts

Experimental approach

The experiments on insect guts were designed to examine large numbers of gut cells. It was therefore decided to use the entire length of the midgut for immunofluorescence, thus increasing the chance of detecting even low levels of infection. This approach has not been documented before and involved the dissection of insects, fixing, staining and mounting of entire intestines. One potential problem, the inclusion of the gut lumen in the mounted gut (Figure 6-3) was addressed by the use of a confocal microscope. Confocal microscopy makes it possible to determine the precise level of origin of the signal within the sample, and therefore could be employed to differentiate between signals originating from the gut lumen and signals originating from the gut cells. Three different methods of visualising cell membranes within the insect gut were tested. These were staining of the cytoskeleton with anti-tubulin antibodies, demarcation of cell surface glycoproteins with wheat germ agglutinin (WGA) and labelling of actin with phalloidin.

Figure 6-3: Schematic cross-section through a Culicoides gut mounted for immunofluorescence microscopy
Infection, maintenance and dissection of Culicoides insects

Batches of *C. sonorensis* (VC) and *C. nubeculosus* insects were fed infectious blood meals containing AHSV-8wt (6.75 log$_{10}$TCID$_{50}$/ml) or AHSV-6wt (6.5 log$_{10}$TCID$_{50}$/ml) as described in section 2.3.2 (page 56). Further batches of *C. sonorensis* (VC), *C. sonorensis* (NVC) and *C. nubeculosus* were IT inoculated with AHSV-8wt or AHSV-6wt, using the technique set out in section 2.3.4 (page 58). Insects were maintained for the duration of the EIP (1, 2 or 10 days) as described in section 2.3.2, anaesthetised with CO$_2$ and dissected immediately. The entire intestinal tract was removed, rinsed with PBS and fixed as described in 2.5.16 (page 65). It was then stored at 4°C for up to five days before being stained using the methods described in section 2.5.17 (page 65). Samples were stained with one of Oregon Green-Phalloidin, anti-tubulin antibody or Texas Red-WGA, with or without PAb-VP7, as indicated in figure legends.

6.2.5 Western blots with anti-NS3/3a antibodies

Western blots were carried out to test the binding characteristics of anti-NS3/3a antibodies with denatured antigen, using cell lysate from infected and uninfected KC cells. Standard protocols were used (section 2.5.18, page 66).

6.3 Results

6.3.1 Visualisation of VP7 and NS3/3a in *C. sonorensis* (KC) cells

The majority of KC cells, when infected with AHSV, were seen to contain hexagonal structures after staining with PAb-VP7 (Figure 6-4 and Figure 6-5). These structures were not observed in uninfected control cells. This is the first time that VP7 crystals have been observed in live cells, rather than after purification of cell lysates.

Staining with the seven anti-NS3/3a antibodies (PAb-R1 and -R2, -G1 to -G5) raised as a part of this project exposed no observable differences between the infected and uninfected KC cells (images not shown). All cells exhibited diffuse staining of the nucleus, cytoplasm and sometimes the cell membrane. This was also the case with the anti-NS3/3a PAb-SA. Variation of antibody concentrations between 1/100 and 1/5000 failed to elicit differences between the infected and uninfected cells.
Staining of infected and uninfected KC cells with the polyclonal anti-NS3/3a peptide PAb-1-117 is shown in Figure 6-4 and Figure 6-5. This method repeatedly identified filamentous structures in AHSV-4-infected KC cells (red). Figure 6-4 also shows staining of the cell membrane, but this could not always be demonstrated in repeat experiments (Figure 6-5). Uninfected cells never showed filamentous structures but did show a high level of nuclear, perinuclear and cytoplasmic background staining with PAb-1-117 (Figure 6-4).
Figure 6-4: Hexagonal and filamentous structures in AHSV-infected KC cells (I)

**Top:** AHSV-4 infected KC cells  
**Bottom:** Uninfected KC cells

KC cells were grown on glass cover slips and infected or mock-infected with AHSV-4, incubated at 29°C for 20 h, fixed with paraformaldehyde and permeabilised with Triton X-100. Incubation with guinea pig-anti PAb-VP7 (1/1000) and rabbit-anti PAb-1-117 (1/500) was followed by incubation with anti-guinea pig antibody 488 nm (green) and anti-rabbit antibody 594 nm (red). Samples were mounted with Vectashield mounting medium and sealed with nail polish.
Figure 6-5: Hexagonal and filamentous structures in AHSV-infected KC cells (II)

The three images show KC cells infected with AHSV-4, stained with anti-NS3/3a PAb-1-117 (red), anti-VP7 antibody PAb-VP7 and DNA marker TO-PRO-3 (blue). The bottom image shows the same VP7 crystal as the top right image at a higher magnification, but without the blue DNA label. Intensities of red and green were reduced in the top right image to enhance the (weaker) blue signal, thus leading to slight colour aberration for red and green in this image.

KC cells were grown on glass cover slips and infected with AHSV-4, incubated at 29°C for 20 h, fixed with paraformaldehyde and permeabilised with Triton X-100. Incubation with guinea pig-anti PAb-VP7 (1/2000) and rabbit-anti PAb-1-117 (1/500) was followed by incubation with anti-guinea pig antibody 488 nm (green), anti-rabbit antibody 594 nm (red) and the TO-PRO-3 (blue). Samples were mounted with Vectashield mounting medium and sealed with nail polish.
6.3.2 Immunofluorescence studies in *Culicoides* midguts

*Visualisation of Culicoides midguts with Oregon Green-Phalloidin, Texas Red-WGA and anti-tubulin antibody*

Figure 6-6 shows that the actin-binding Oregon Green-Phalloidin is suitable for visualising the midgut of *Culicoides* insects. The structure highlighted by this stain probably represents the extensive smooth muscle network of the insect midgut. The computer-generated z-section (a vertical cross-section through the mounted sample) shows that phalloidin does not stain all gut cells and is therefore not suitable for outlining the luminal and abluminal borders of the midgut.

Figure 6-7 shows that both anti-tubulin antibody and lectin-binding WGA will stain midgut cells. The computer-generated z-sections suggest that the anti-tubulin antibody may not penetrate the entire gut wall while WGA appears to penetrate the gut walls very well, showing the opposing gut walls compressing the gut lumen.

These results suggest that WGA is the most suitable stain for outlining the gut cell margins, which would help to identify whether other signals originate from the intracellular space, the cell surface or the gut lumen. However, the quality of the WGA staining could not be reliably reproduced as shown in this experiment. Phalloidin and anti-tubulin antibody were therefore used in subsequent experiments.
Figure 6-6: Visualisation of *C. sonorensis* midgut with phalloidin

**Top left:** Whole midgut; **Top right:** enlarged section of posterior midgut; **Bottom:** Computer-generated z-section through posterior midgut. The figure shows the phalloidin-stained smooth muscle network.

Figure 6-7: Visualisation of *C. sonorensis* midgut with anti-tubulin antibody and WGA

Posterior midgut surface stained with anti-tubulin antibody (**top left**) and WGA (**top right**). Computer-generated z-sections of these areas (**bottom**) show the gut walls enclosing the gut lumen (**→** arrows).

Adult female *C. sonorensis* were anaesthetised, dissected and their entire intestines removed. These were then fixed with paraformaldehyde, permeabilised with Triton X-100 and stained with Oregon Green-Phalloidin, mouse anti-tubulin antibody/anti-mouse 594 nm antibody or Texas Red-WGA, then mounted with Vectashield mounting medium, sealed with nail polish and examined under a confocal microscope.
Identification of VP7 crystals in Culicoides midguts

Figure 6-8 shows that IT inoculation of *C. sonorensis* with AHSV-8wt results in the presence of characteristic VP7 crystals in the midgut. IT inoculation was chosen as a route of infection to ensure that (almost) all insects were infected, so that the ability of the test to detect VP7 crystals could be properly assessed.

The VP7 crystals are concentrated in the posterior part of the midgut but are also common in the anterior midgut. The z-section strongly suggests that the crystals are located in the gut wall, i.e. the midgut cells. This suggests that they are not artefacts originating from residual virus from the IT inoculation but are the result of virus replication in the gut cells. To confirm that the breakdown of virus does not lead to detectable VP7 crystals in the absence of virus replication, a biological negative control was carried out (below).
Figure 6-8: Infected *C. sonorensis* (VC) midgut contains VP7 crystals in the gut wall

**Top:** Entire midgut with Malphigian tubules and midgut-hindgut junction.

**Bottom left:** Enlarged VP7 crystals in posterior midgut.

**Bottom right:** Computer-generated z-section through posterior midgut.

3-day-old adult female *C. sonorensis* (VC) were IT inoculated with AHSV-8wt. After an EIP of 10 days they were anaesthetised, dissected and their entire intestines removed. These were then fixed with paraformaldehyde, permeabilised with Triton X-100 and incubated with PAb-VP7 and mouse anti-tubulin antibody, followed by incubation with anti-guinea pig antibody 488 nm (green) and anti-mouse 594 nm antibody (red), then mounted with Vectashield mounting medium and sealed with nail polish.
Biological negative control

Figure 6-9 shows that feeding of an AHSV-8wt-containing blood meal to the orally refractory species *C. nubeculosus* did not result in the formation of VP7 crystals after an incubation period of 2 days. Similarly, no VP7 crystals were detected after incubation periods of 0 and 1 days, suggesting the absence of AHSV in each case (images not shown). The results of IT injection of *C. nubeculosus* with AHSV-6wt are shown in the following section.
Figure 6-9: VP7 crystals are absent from the midguts of orally-refractory C. nubeculosus insects two days after a blood meal containing AHSV

Top: Midgut with midgut-hindgut junction (right).
Bottom: Enlarged part of posterior midgut.

Green: Midgut smooth muscle network.
Red: AHSV VP7 (none identified).

3-day-old adult female C. nubeculosus were fed an infectious blood meal of AHSV-8wt. After an incubation period of 2 days they were anaesthetised, dissected and their entire intestines removed. These were then fixed with paraformaldehyde, permeabilised with Triton X-100 and incubated with guinea-pig, anti-VP7 PAb-VP7, followed by incubation with anti-guinea pig antibody 594 nm (red) and staining with Oregon Green-Phalloidin (green). Samples were then mounted with Vectashield mounting medium and sealed with nail polish.
Replication of AHSV-8wt and AHSV-6wt in Culicoides midguts following oral infection and IT inoculation

Confocal microscopy was not available at IAH Pirbright and disease security regulations caused additional logistical problems in transporting such samples to a location where a confocal microscope was sited. Therefore, following the experiments above, which showed that the presence of VP7 in Culicoides midguts indicates virus replication, samples were stained with Oregon Green-Phalloidin and PAb-VP7 and checked for the presence of VP7 crystals under a non-confocal microscope. The results in Table 6-2 show that the feeding of infectious blood meals containing AHSV-8wt results in a midgut infection of most *C. sonorensis*, but none of the *C. nubeculosus* insects were detectably infected. In contrast, the feeding of AHSV-6wt didn’t result in a detectable midgut infection in either of these two species.

Table 6-2 also shows that IT inoculation of AHSV-6wt results in a midgut infection in *C. sonorensis* (VC and NVC) but not in *C. nubeculosus*. IT inoculation of AHSV-8wt into *C. sonorensis* (VC) also resulted in a midgut infection.

Table 6-2: Presence of VP7 in Culicoides midguts following oral infection and IT inoculation with two AHSV strains

<table>
<thead>
<tr>
<th>AHSV strain</th>
<th>Number of insects positive/number tested</th>
<th>Fed Orally</th>
<th>Inoculated IT</th>
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<tr>
<td></td>
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<td><em>C. sonorensis</em> (VC)</td>
<td><em>C. sonorensis</em> (VC)</td>
</tr>
<tr>
<td>8wt</td>
<td></td>
<td>5/6</td>
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<td></td>
<td>0/6</td>
<td>8/8</td>
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</tbody>
</table>

¹ n.d.: not done

6.3.3 Western blot analysis of the anti NS3/3a PAb-1-117

The anti-NS3/3a antibody PAb-1-117 was used in Western blots in an attempt to establish whether the filamentous structures identified with this antibody in AHSV-4 infected KC cells (section 6.3.1) were due to binding of NS3/3a, or the result of cross-reactions with other proteins. Figure 6-10 shows that the antibody binds to three
proteins, with sizes of approximately 15, 25 and 45-50 kDa. The 25 kDa protein is not visible in the negative control and has the expected size of NS3/3a. The 15 and 45 kDa proteins are thought to be cellular proteins since these bands also appear in uninfected cells.

Figure 6-10: Western blot analysis of AHSV-4 infected KC cell lysate with anti-NS3/3a PAb-1-117

AHSV-4 infected KC cells were lysed 24 h post infection, denatured at 100°C and resolved alongside a broad range protein marker by SDS-PAGE. Proteins were transferred onto an Immobilon P membrane and blocked in 5% Marvel (in TBS Tween) overnight. PAb-1-117 was added at 1/1000 and incubated for 1 h. 4 TBS/Tween washes were followed by incubation of the secondary antibody (anti-rabbit peroxidase-linked antibody, 15 µl/10 ml blocking buffer) for 1 h and further washes as before. All incubation was at room temperature under constant rotation. Development was with Luminol enhancer and Stable Peroxide (Pierce) for 1 min, followed by immediate exposure to film.

6.4 Discussion

The work described does not entirely reflect the original objectives of the present chapter. To investigate how NS3/3a may affect virus spread within the midgut, or release from the midgut into the haemocoel, the first objective was to localise NS3/3a in infected KC and C. sonorensis midgut cells, and compare the results between the two reassortant viruses. The second objective was to test the hypothesis that vector competence of the C. sonorensis VC colony is linked to the type of NS3/3a present (see chapter 4) by localising NS3/3a in C. sonorensis VC and NVC midgut cells, and comparing the results for the two colonies. This approach met with two obstacles.
The first obstacle was that the difference in the ability of AHSV-A790 and AHSV-A79 to escape from host cells is quantitative in nature. In KC and BHK-21 tissue culture, both viruses are released into the supernatant, although AHSV-A79 regularly grows to higher titres in KC cells (chapter 7). Similarly, in C. sonorensis insects, both viruses have the potential to be released from the abluminal midgut cell membrane (chapter 4). Since this is true for both the NVC and the VC insect colony, there is no virus-host system that always allows the virus to be released into the haemocoel and no system that always prevents virus release. Consequently, single observations in immunofluorescent microscopy cannot be firmly associated with the release or non-release of the virus from the observed cell. However, immunofluorescent microscopy, especially of entire insect guts, is not well suited to large scale repetition to provide statistically significant numbers of observations.

The second obstacle towards association of different cellular NS3/3a-localisations with release or non-release events was the lack of anti-NS3/3a antibodies.

Identification of infected KC and Culicoides midgut cells

The results of this chapter show that AHSV infection of KC cells and Culicoides midgut cells leads to the formation of cytoplasmic VP7 crystals. The significance of this finding is unclear but may reflect overexpression of VP7. The experiments also show that the anti-VP7 antibody PAb-VP7 can be used successfully as a control to detect AHSV-infected cells. Staining of Culicoides guts with anti-tubulin, WGA or phalloidin did not consistently succeed in outlining the margins of individual cells but the “biological negative control” (Figure 6-9) detected no VP7 crystals in C. nubeculosus guts. This shows that the presence of these crystals in Culicoides guts is a sign of AHSV replication, rather than an indicator of residual virus protein from the infectious blood meal. The location of the VP7 crystals in the insect wall of Figure 6-8 also supports the view that that these crystals are produced during virus replication in the gut cells and are not due to the presence of degraded virus particles in the gut lumen (following oral infection) or the haemocoel (following IT inoculation).

Assessment of anti-NS3/3a antibodies

Immunofluorescence microscopy in KC cells revealed no significant differences between AHSV-infected and uninfected cells when analysed with the NS3-antipeptide
antibodies (PAb-R1 and -R2, -G1 through -G5) or PAb-SA. It is possible that the target region of these antibodies is an internal structure and is thus not available for antibody binding unless the protein is denatured. Western blot analysis of these antibodies showed bands at approximately 25 kDa (the size of NS3/3a, figures not shown) but proved inconclusive due to the simultaneous presence of several other bands in infected and uninfected cells, suggesting cross-reaction of the antibody with cellular proteins. Binding of the antibodies to both nuclear and cytoplasmic structures of infected and uninfected cells in immunofluorescence studies also suggest the presence of cross-reaction between NS3-antipeptide antibodies and cellular proteins.

Labelling of AHSV-infected KC cells with PAb-1-117 revealed filamentous structures that were not present in uninfected control cells. However, the role of NS3/3a as a cell membrane protein does not easily explain this finding. Western blot analysis of KC cells with this antibody showed binding to an NS3/3a-sized protein in infected, but not in uninfected cells, suggesting that it does bind to NS3/3a. The binding of the antibody to 15 kDa and 45 kDa proteins in infected and uninfected KC cell lysates suggests the presence of cross-reactions of the antibody with cellular proteins. These cross-reactions might not occur to the same extent in immunofluorescence microscopy since some of the antigenic sites may only be accessible to the antibody in denatured proteins. The size of the bigger cellular protein interacting with PAb-1-117 in Western blot analysis suggests that this protein could be actin, which is an important part of the cytoskeleton. If this is the case then the structures seen in infected cells would suggest that infection with AHSV leads to the formation of so called actin stress fibres.

Susceptibility of Culicoides midgut cells to AHSV infection from the luminal and the abluminal side

The absence of VP7 crystals in C. nubeculosus midguts following oral ingestion of AHSV-8wt or AHSV-6wt (Table 6-2, page 139) strongly suggests that virus replication does not take place in C. nubeculosus midgut cells even at a level too low to be detected with the virus infectivity assay. It is therefore likely that the failure of C. nubeculosus to become orally infected with AHSV is due to a failure of the virus to enter and replicate in the midgut cells. In combination with the absence of infection from the abluminal side in the same species, these data suggest that C. nubeculosus midgut cells are resistant to infection with AHSV. Whether the midgut cells prevent the
virus from entering or permit virus entry but not replication, remains unanswered. The failure of *C. nubeculosus* midgut cells to become infected from the ab luminal side sets this refractory insect species apart from both the VC and the NVC colonies of *C. sonorensis*. The fact that the midgut cells of both *C. sonorensis* colonies could be readily infected from the ab luminal side suggests fundamentally different mechanisms underlying the very low susceptibility (reflected by 0 oral infection rates in the extreme) of some populations of essentially vector-competent species (e.g. the NVC *C. sonorensis* colony) and the resistance of non-vector species such as *C. nubeculosus*.

The ability of AHSV-6wt to infect *C. sonorensis* midgut cells from the ab luminal side shows that both laboratory colonies of this species, irrespective of oral susceptibility to AHSV, possess AHSV receptors on the ab luminal side of their midguts. This result supports the view that it is possible for AHSV particles to spread from one midgut cell to another via the haemocoel, as has been suggested in chapter 4. It also supports the opinion that selection of the VC and NVC *C. sonorensis* colonies did not affect the ab luminal cell membrane (representing the MEB). Furthermore, the replication of AHSV-6wt in *C. sonorensis* midgut cells following IT inoculation shows that the failure of AHSV-6wt to infect these cells from the gut lumen represents a failure of the virus to enter the cell. This is most likely due to an inability of this virus strain to utilise the same receptors used successfully by other virus strains, such as AHSV-8wt and AHSV-A79.
7 Growth characteristics of AHSV in C. sonorensis (KC) cells

7.1 Introduction

The earlier parts of the present work showed that variation in AHSV genome segment 10 can affect oral infection rates in the model vector species C. sonorensis (chapter 4) and the field vector species C. imicola (chapter 5). The identity of segment 10 was also shown to have an effect on AHSV dissemination through the body of C. sonorensis, thus affecting vector competence (chapter 4). These data were obtained using the reassortant virus pair AHSV-A790 and AHSV-A79. The two viruses were originally produced by co-infection of BHK-21 cells with the parent virus strains AHSV-3att and AHSV-8wt (O'Hara, 1994). AHSV-A790 shares segments 1-6 and 8 with AHSV-8wt and segments 7, 9 and 10 with AHSV-3att. AHSV-A79 shares segments 1-6, 8 and 10 with AHSV-8wt and segments 7 and 9 with AHSV-3att (O'Hara, 1994). AHSV-A790 and AHSV-A79 therefore only differ in genome segment 10.

The first aim of this chapter was to acquire preliminary data to establish whether the different oral infection rates and dissemination rates in vector insects with the two reassortants could be based on differences in virus release rates from the host cells. Experiments with BTV VLPs in baculovirus expression systems indicate that NS3/3a (encoded by genome segment 10) is essential for virus exit from insect cells (Hyatt et al., 1993). The presence of ubiquitin-ligase recruiting motifs in AHSV NS3/3a also suggests a role for this protein in virus exit from the cell (section 3.2.3, page 72). These motifs have been found to be essential in several retroviruses for budding to take place (Strack et al., 2000). Since budding is the principal orbivirus exit mechanism in C. sonorensis (KC) insect cell culture (Fu, 1996) and this cell line is closely related to the model vector insects used in chapters 4 and 6, KC cells were used in this study. They do not undergo lysis or show CPE when infected with AHSV or related orbiviruses.

The second aim of the work described in this chapter was to verify whether AHSV-6wt replicates in KC cells. This virus strain has been routinely grown in mammalian (BHK-21) cells but C. sonorensis and C. imicola have been found to be refractory to infection with this virus by the oral route (Wittmann, 2000). It did, however, infect two C. bolitinos (equalling 2% IR) by the oral route, and replicated in C. sonorensis following IT inoculation.
To address the stated aims, two experiments were carried out. In the first experiment, growth curves were generated, analysing the titres of the two reassortant viruses and their two parent strains in KC tissue culture supernatants. In the second experiment, flasks of KC cells were infected with one of the four virus strains, or with AHSV-6wt. The amount of virus that was retained intracellularly or released into the supernatant was measured in each case and used to calculate the proportion of virus released from the cells at each time point.

7.2 Methods

AHSV growth curves in KC tissue culture supernatant

Four 25 cm² tissue culture flasks, containing confluent monolayers of KC cells, were each infected with 4.25 log₁₀TCID₅₀/ml supernatant (7 ml) of one of AHSV-3att, AHSV-A790, AHSV-A79 or AHSV-8wt. The virus was gently removed after 1 h and replaced with an equal volume of tissue culture medium. The flasks, each containing 7 ml of tissue culture supernatant, were incubated at 29±1 °C and 200 µl supernatant from each flask was removed at regular intervals. These intervals progressively increased from initially 2 h to over 24 h at the end of the six day experiment. The samples were stored at -70°C and later assayed using the virus infectivity assay described in section 2.2.4 (page 52).

Linear regression was carried out for the exponential part of some virus growth curves (0-84 h pi). 95% confidence intervals were calculated from the standard errors of the regression and plotted with the regression lines. This allowed comparison of the speeds at which virus strains accumulated in the supernatant. The limitations of the data obtained and the consequences of this for the data interpretation are discussed in section 7.4 (page 152).

Retention and release of AHSV from KC tissue culture

25 cm² tissue culture flasks, containing confluent KC cell monolayers, were infected with one of five virus strains. These were the reassortant and parent virus strains used in the experiment described above and AHSV-6wt. Each strain was used to infect four flasks with 3 log₁₀TCID₅₀ of virus each. Virus-containing supernatant was removed after 1 h and replaced as described above. The infected flasks were incubated
at 29±1°C. One flask of each strain was removed at 3, 24, 48 and 120 h post infection. Flasks did not show an obvious increase in cell numbers over this time period. The supernatants were carefully removed to avoid detaching cells from the cell sheet. Supernatants were then centrifuged for 5 min at 700 g to remove cells and cell debris. They were stored at -70°C until assayed for AHSV. The cell sheet was rinsed carefully with PBS three times to remove remaining supernatant, suspended in a volume of tissue culture medium equal to that of the removed supernatant (7 ml) and sonicated for one minute to break up the cells and release intracellular virus particles. Samples were then stored at -70°C until assayed. Absolute amounts of intracellular and extracellular virus (per flask) were calculated from the measured virus concentrations and the volumes of tissue culture medium used. The total amount of virus synthesised in each tissue culture flask was calculated from the absolute amounts of intra- and extracellular virus. These values were used to determine the proportions of synthesised virus released into the supernatant with each virus strain.
7.3 Results

**AHSV growth curves in KC tissue culture supernatant**

Table 7-1: Titres of four AHSV strains in KC tissue culture supernatant

<table>
<thead>
<tr>
<th>Hours post infection</th>
<th>3att</th>
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<th>A79</th>
<th>8wt</th>
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<tr>
<td>0</td>
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Four 25 cm² tissue culture flasks, containing confluent monolayers of KC cells, were infected with 4.25 (7 ml) log_{10} TCID_{50}/ml of one of AHSV-3att, AHSV-A790, AHSV-A79 and AHSV-8wt. The flasks were incubated at 29±1°C. The supernatant from each flask was sampled at regular intervals and assayed for AHSV by virus infectivity assay.

The development of virus titres from 0 to 146 hours pi in tissue culture supernatant is shown in Table 7-1 and illustrated in Figure 7-1 (page 148). The four curves suggest that virus growth was exponential until about 84 hours pi when it began
to plateau. AHSV-A79 and AHSV-8wt reached end titres approximately 1 log$_{10}$TCID$_{50}$/ml higher than those reached by the other two virus strains. No viral eclipse was observed. Linear regression was carried out for the exponential growth period from 0 to 84 hours pi (Figure 7-2 top, page 149). Figure 7-2 (bottom) shows 95% confidence intervals for the regression lines. The equations for the regression lines and their confidence intervals are also included. Figure 7-2 shows that, in this experiment, the two reassortant viruses accumulated in KC tissue culture supernatant at significantly different speeds.

**Figure 7-1: Growth curves of four AHSV strains in KC cell tissue culture supernatant**

Four 25 cm$^2$ tissue culture flasks, containing confluent monolayers of KC cells, were infected with 4.25 log$_{10}$TCID$_{50}$/ml (7 ml) of one of AHSV-3att, AHSV-A790, AHSV-A79 and AHSV-8wt. The flasks were incubated at 29±1°C. The supernatant from each flask was sampled at regular intervals and assayed for AHSV by virus infectivity assay.
Figure 7-2: Linear regression for two reassortants (top) and their regression lines with 95% confidence intervals (bottom)

X: time (hours)  
Y: virus titre (log₁₀TCID₅₀/ml)

Tissue culture flasks containing confluent monolayers of KC cells were infected with 4.25 log₁₀TCID₅₀/ml supernatant of AHSV-A790 or AHSV-A79 and incubated at 29±1°C. The supernatant from each flask was sampled at regular intervals and assayed for AHSV. Regression lines and their 95% confidence intervals were calculated.
Retention and release of AHSV from KC tissue culture

The results in Table 7-2 show that the five AHSV strains replicated to final supernatant titres between 6.35 (AHSV-A790) and 8.35 (AHSV-A79) log_{10}TCID_{50}/flask (a 100-fold difference between these virus strains). Final titres of intracellular virus ranged from 6.35 (AHSV-A790) to 8.1 (AHSV-A79) log_{10}TCID_{50}/flask (a 56-fold difference). The growth curves for released and intracellular virus are illustrated in Figure 7-3 (top, page 151). Growth curves for total virus (calculated from supernatant and intracellular virus) are also shown (Figure 7-3, bottom). Figure 7-3 illustrates that, in this experiment, AHSV-A79 reached higher intra- and extracellular titres than AHSV-A790 and that the absolute replication level of AHSV-A79 was also higher than that of AHSV-A790. Figure 7-3 also shows that AHSV-6wt replicates in KC cells. Titres of AHSV-6wt were all within the range established by the other virus strains.

Since the intracellular and extracellular virus titres are functions both of virus replication levels and virus release efficiency, Figure 7-3 does not clarify whether the observed differences between the virus strains indicate a difference in the efficiency of virus release from the cell. Quantities of released virus are therefore illustrated as proportions of the total virus titre in Figure 7-4 (page 152). The figure shows that virus release into the supernatant was more efficient in AHSV-A79 than in AHSV-A790 at all four time points, but especially so at 24 and 48 h post infection. Values for the parent virus strains are included for comparison.

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Figure 7-3: AHSV release from KC cells (top left), intracellular retention (top right) and total virus synthesised (bottom)

Confluent KC cell monolayers in 25 cm² tissue culture flasks were infected with 3 log₁₀TCID₅₀ AHSV (one of AHSV-A790, AHSV-A79, AHSV-8wt, AHSV-3att or AHSV-6wt). Four flasks per virus strain were incubated at 29±1°C and 1 flask per virus strain was removed at 3, 24, 48 and 120 h post infection. The supernatants were removed and centrifuged for 5 min at 700 g, then stored at -70°C until assayed for AHSV. The cell sheet was rinsed with PBS 3 times, suspended in a volume of tissue culture medium equal to that of the removed supernatant (7 ml) and sonicated for one minute to break up the cells and release intracellular virus particles. Samples were then stored at -70°C and later assayed. Supernatant and intracellular virus quantities per flask were calculated from the measured concentrations and the volume used. Total virus quantities were calculated from extracellular and intracellular virus quantities.
Confluent KC cell monolayers in 25 cm² tissue culture flasks were infected with 3 log₁₀TCID₅₀ AHSV (one of AHSV-A790, AHSV-A79, AHSV-8wt or AHSV-3att). Four flasks per virus strain were incubated at 29±1°C and 1 flask per virus strain was removed at 3, 24, 48 and 120 h post infection. The supernatants were removed and centrifuged for 5 min at 700 g, then stored at -70°C until assayed for AHSV. The cell sheet was rinsed with PBS 3 times, suspended in a volume of tissue culture medium equal to that of the removed supernatant (7 ml) and sonicated for one minute to break up the cells and release intracellular virus particles. Samples were then stored at -70°C and later assayed. Supernatant and intracellular virus quantities per flask were calculated from the measured concentrations and the volume used. Total virus quantities were calculated from extracellular and intracellular virus quantities.

7.4 Discussion

In the first experiment described in this chapter, AHSV-A79 accumulated in KC tissue culture supernatant faster and reached higher titres than AHSV-A790. Due to time constraints, the experiment was carried out with one flask per virus strain only. Consequently, only one measurement existed for each time point and there was no indicator of the degree of natural variation in the growth curves (i.e. it is unknown how much virus titres in duplicate flasks would have diverged from the presented results). Analysis of these data must therefore be based on the assumption that the recorded data represent the true values, i.e. duplicate experiments would have registered exactly the same results. This would in all likelihood not be the case. Therefore, the statistically significantly different regression lines between the two reassortant virus strains (Figure 7-2, page 149) apply to the described experiment only and may not be confirmed on more intensive study. However, the similarity of the shown results with those of the second experiment (Figure 7-3) and with previous observations (when growing AHSV strains in KC cells for other purposes, results not shown) suggest that the results are
representative and based on a real phenomenon. This should be verified by further work, carried out with four to five replicate flasks per virus strain. The sensitivity of such an experiment to variation between virus strains could also be enhanced by increasing the number of replicate wells used in the virus infectivity assay (section 2.2.4, page 52) from four to, say, ten wells per sample. This would reduce the lowest measurable difference between two virus titres from $0.25 \log_{10} \text{TCID}_{50}/\text{ml}$ to $0.1 \log_{10} \text{TCID}_{50}/\text{ml}$.

In the second experiment, AHSV-A79 again replicated faster and to higher titres than AHSV-A790. Ratios between released and intracellularly retained virus quantities allow speculation that AHSV-A79 possesses a more effective release mechanism than AHSV-A790 and such a finding could explain the higher infection and dissemination rates with AHSV-A79 in Culicoides vector insects (chapters 4 and 5). However, the limitations described for the first experiment of this chapter also apply here. Additionally, derivation of decimal numbers for calculation of ratios from values that were measured on a logarithmic scale makes these ratios extremely sensitive to variations in the measured (logarithmic) titres.

Variation in genome segment 10 may affect AHSV growth curves in two ways. Firstly, an increased rate of virus escape from the host cell will aid rapid virus spread through the tissue culture and accelerate the infection of all susceptible cells in the flask. Secondly, a more efficient virus release mechanism will enhance superinfection of (already infected) cells, thus making these cells more productive (Hyatt et al., 1989). Progeny virus particles cannot replicate while being retained in the cell since the possession of outer capsid proteins VP2 and VP5 renders them inactive (section 1.2.4, page 30).

It can only be speculated which structural differences between the NS3/3a proteins of the reassortant virus strains could be responsible for the differences seen in the two experiments described in this chapter. NS3/3a’s of AHSV-A79 and AHSV-A790 possess an identical ubiquitin ligase recruiting motif in the same position. Differences in this motif could affect virus budding from host cells (Strack et al., 2000), although it has not yet been shown to be active in orbiviruses. The two virus strains differ in myristylation (membrane anchoring) motifs and may also differ in their transmembrane configuration (section 3.2.3, page 72). Differences in membrane anchors could result in different efficiencies of membrane targeting while different transmembrane
arrangements could expose or obscure yet unidentified protein domains from interaction with other viral or host cell proteins. Alternatively, differences between the reassortants may result from other, yet unidentified motifs. The discovery of further, function-defining motifs in NS3/3a is not unlikely since its mode of action has not yet been defined.

The second experiment described in this chapter also shows that AHSV-6wt grows to high titres in the *C. sonorensis*-derived KC cell line. This virus also replicates in the BHK-21 cells used in all virus infectivity assays. This rules out the hypothesis that AHSV-6wt may be defective and for this reason is unable to infect *C. sonorensis* or *C. imicola* by the oral route. Such a hypothesis also seems unlikely in light of the finding that AHSV-6wt replicates in *C. sonorensis* following IT inoculation (section 4.3.4, page 97). The present result supports the suggestion that this virus strain is unable to penetrate the midgut infection barriers of its potential vector species. This may be caused by a different VP2, which may require a different cell surface receptor to other AHSV strains tested. This receptor would be absent on the luminal surface of the *C. sonorensis* midgut cells, but present on their abluminal surface and on the surface of KC tissue culture cells. Appropriate receptors for the binding of VP2 of AHSV-6wt might be present on the luminal midgut cell surface of other *Culicoides* populations/species, allowing them to become infected and act as vectors.

AHSV-A790 and AHSV-3att possess the same segment 10 and the virus pair AHSV-A79 / AHSV-8wt are also identical in this genome segment. Therefore, it might seem logical to expect similar results (in both of the described experiments) for these respective virus pairs. However, these viruses differ in several other genome segments, which may also have an effect on virus titres through their role in host cell infection, virus replication, packaging etc.
8 Conclusions and future work

8.1 Summary of Conclusions

- Variation in AHSV NS3/3a affects oral infection rates with the virus in the model vector *C. sonorensis* and the field vector *C. imicola*. It also affects disseminated-infection rates in *C. sonorensis*. These findings illustrate the potential of NS3/3a variation to have an effect on AHSV transmission in the field.

- Variation in AHSV NS3/3a affects the virus dissemination from midgut cell to midgut cell, and from the midgut cells into the haemocoel of *C. sonorensis*.

- Differences in membrane association of NS3/3a variants could be responsible for the different biological properties of the two reassortant viruses AHSV-A79 and AHSV-A790.

- The original selection process for *C. sonorensis* vector competence for AHSV was, at least in part, a selection for successful interaction with an α-group NS3/3a. It led to increased insect susceptibility to at least one other AHSV strain with an α-group NS3/3a.

- The *C. sonorensis* mesenteronal infection barrier to AHSV is insect- and virus-defined. It can be modified by insect selection for and against vector competence.

- The *C. sonorensis* mesenteronal escape barrier to AHSV is mainly virus-defined and is not affected by insect selection for and against vector competence.

- Midgut cells of an insect belonging to a *C. sonorensis* colony with low vector competence will usually replicate AHSV if infection takes place from the abluminal midgut membrane. Conversely, midgut cells of the non-vector species *C. nubeculosus* do not replicate AHSV under these conditions. This qualitative difference could be used to differentiate between potential vector species and non-vector species of AHSV and, potentially, other orbiviruses.

- Heavily attenuated AHSV strains can infect their field vectors *C. imicola* and *C. bolitinos*. This raises the question whether live attenuated virus vaccines may also be able to infect *Culicoides* insects, which could lead to virus spread, reversion to virulence and reassortment with field virus strains.
Chapter 4 showed that the identity of AHSV NS3/3a significantly affects oral infection rates with the virus in *C. sonorensis* insects. Similarly, variation in this protein affected oral infection rates in the main field vector *C. imicola*. These findings demonstrate that NS3/3a can have a major effect on the spread of AHSV, and, therefore, the epidemiology of AHS. By implication, the large extent of variation in this protein may, in combination with reassortment, be an important factor in virus evolution and adaptation to insect populations and species.

The possible role of variation in AHSV NS3/3a in virus adaptation to different insect vectors begs the question why similar degrees of variation have not been found in NS3/3a’s of other *Orbivirus* species. The apparent lack of variation may, in part, be explained by the limited amount of sequencing data available for these viruses. Additionally, the life cycles of orbiviruses remain relatively poorly understood and these viruses may vary in their precise cell exit mechanisms.

Analysis of infection rates and disseminated-infection rates in orally infected *C. sonorensis* showed that NS3/3a affects virus dissemination from one midgut cell to another in the VC colony only, while it had an effect on virus dissemination from the midgut to the haemocoel in both the VC and the NVC colonies. The most logical explanation for the different effects of variation in NS3/3a in the two colonies would be that the AHSV-9 that was used in the original selection process for vector competence, possesses a type of segment 10 similar to the one found in AHSV-8wt and AHSV-A79 (the virus strains which achieved high infection rates in the VC colony). The results of sequencing work carried out previously in the Orbivirus group confirm that the AHSV-9 used in the selection process possesses an α-group segment 10, as do AHSV-8wt and AHSV-A79 (accession number AJ007308, Martin *et al.*, 1998). This means that the original selection of the VC colony was, at least in part, a selection for an α-group NS3/3a.

It appears likely that virus spread within the midgut (which modified vector competence) of a *Culicoides* insect is affected by a factor (e.g. a protein) that is unique to the vector-competent colony and interacts with AHSV NS3/3a. This hypothetical factor does not interact equally efficiently with all types of this viral protein. The molecular basis of how variation in NS3/3a affects virus dissemination through the
insect vector remains unclear, but analysis of the two reassortant virus NS3/3a protein sequences showed possible differences in their transmembrane configuration. They also differed in some myristylation (membrane anchoring) motifs.

Future work should concentrate on the exploration of these protein regions since this could help to establish the mode of action of NS3/3a. It could also shed light on the nature of virus dissemination barriers in the insect vector (see below). In the absence of virus rescue systems, approaches could include the mutation of the two NS3/3a variants and their expression in baculovirus expression systems (for more detailed suggestions, see page 79). Such an approach could verify the significance of the potential myristylation motifs in which the two reassortant NS3/3a proteins differ. In addition, mutation of AHSV NS3/3a transmembrane regions has been shown to affect cytotoxicity (van Niekerk et al., 2001a) and could thus be used to verify the suggested differences in membrane configuration between the two reassortant virus NS3/3a’s.

8.3 Midgut barriers to AHSV dissemination in Culicoides insects

Analysis of infection rates and disseminated-infection rates in the VC and NVC colonies of C. sonorensis indicated that selection of these colonies for and against vector competence for AHSV resulted primarily in a modification of the virus ability to infect and colonise the insect midgut (MIB), while the abluminal midgut cell membrane (representing the MEB) remained essentially unchanged. The latter finding was supported by the observation that the midguts of both C. sonorensis colonies can be infected from the abluminal side (following IT inoculation) equally well. This is in contrast with the midgut cells of the (orally refractory) C. nubeculosus, which did not replicate AHSV following attempted infection from either the luminal or the abluminal midgut membrane (chapter 6).

In summary, midgut dissemination barriers of Culicoides vector species can be characterised as follows:

1) The MIB is insect- and virus-defined. It can be modified by selection for and against vector competence. This shows that the MIB is, at least partly, genetically defined. AHSV ability to overcome the MIB is modified by virus protein NS3/3a. This modification apparently controls the efficiency of virus dissemination from one midgut cell to another.
2) The MEB is mainly virus-defined and not affected by selection for or against vector competence. It is modified by NS3/3a. Virus strains that disseminate well within the midgut also disseminate more successfully from the midgut into the haemocoel.

3) There is a qualitative difference between populations of potential vector species with low vector competence (e.g. C. sonorensis NVC colony) and non-vector species (e.g. C. nubeculosus): Midgut cells of an insect belonging to a vector species will usually replicate AHSV if infection takes place from the abluminal midgut membrane, while midgut cells of a non-vector species insect seem unable to do so. The absence of AHSV replication in midgut cells of the non-vector species C. nubeculosus indicates that either these cells are intrinsically unable to replicate the virus, or that the abluminal membrane of the midgut cells cannot be penetrated by AHSV. Since other C. nubeculosus cells are capable of replicating AHSV (section 4.3.4, page 97), the latter of the two possibilities seems to be more likely. The abluminal side of the midgut cells is covered by the basement lamina, which, in C. nubeculosus, may not possess suitable receptors for the attachment and penetration of AHSV particles.

The described difference between C. sonorensis (NVC) and C. nubeculosus could potentially be used as a marker to assess the risk of AHSV transmission by Culicoides insects in regions threatened by outbreaks of AHS. The same difference may apply to the infection of Culicoides insects with other orbiviruses, such as BTV. This means that, if crystallisation of BTV VP7 could be shown to occur in insect cells, Culicoides populations could also be screened for their potential to transmit BTV.

Future work should aim at identifying the genetically determined factor or factors in which the VC and the NVC Culicoides colonies differ. Since the VC colony appears to possess a factor which interacts with NS3/3a (chapter 4), it might be possible to identify such a factor by looking for proteins that interact with this NS3/3a in C. sonorensis midgut cells. Techniques could include immunoprecipitation, yeast-2-hybrid systems and GST fusion assays, using midgut cell lysates from the VC and NVC colonies and comparing the results between them.
8.4 Susceptibility of Culicoides field vectors to infection with attenuated AHSV strains

Chapter 5 showed that heavily attenuated AHSV strains can infect their field vectors *C. imicola* and *C. bolitinos*. This causes concern about the safety of using live attenuated virus vaccines against AHSV and other orbiviruses, such as BTV. Potential risks include vaccine virus reassortment with wild-type virus strains in the mammalian host or insect vector, virus spread by the insect vector and reversion to virulence of vaccine virus strains. A further disadvantage of these vaccines is that they do not allow a distinction to be made between vaccinated animals and those that have been in contact with a field strain of the virus.

While such risks may not matter much in endemic areas, where several virus strains circulate simultaneously and may reassort freely, they could undermine the success of disease control measures in non-endemic areas, which must be aimed at the eradication of the virus from the affected region. Examples include the AHS outbreak on the Iberian peninsula (1987-1990) and the recent outbreaks of BT in parts of southern Europe (since 1998). A more detailed risk analysis of AHS (and BT) live attenuated virus vaccines is therefore desirable and should include the assessment of commercially available vaccine strains for their potential to cause viraemia in the potential target species (horses, mules and donkeys). To assess their potential to revert to virulence, these virus strains could be subjected to multiple alternating passages in mammalian hosts and insect vectors. They should also be included in field-vector competence studies similar to the ones described in chapter 5.

To avoid the risks of live attenuated virus vaccines, ideally only non-replicative vaccines should be used in non-endemic areas. These could include inactivated virus vaccines or subunit vaccines, which contain some or all structural proteins but no genome. A European Union project is currently working towards the development of such vaccines.
9 Appendix I: AHSV NS3 sequence alignment

The following pages show the predicted protein sequences of the AHSV NS3 strains sequenced in this project, aligned to all other available AHSV NS3 protein sequences. Protein sequences obtained from databases are identified by their serotype (ST), followed by their TrEMBL or SwissProt accession number. NS3 isolates belonging to the α group are positioned on top (14 sequences), followed by β group (12 sequences) and then γ group (6 sequences). Amino acid (aa) residues that are conserved across all isolates are highlighted black, residues conserved across the α group are highlighted yellow, residues conserved within the β group are turquoise and those conserved within the γ group are pink.

Potential glycosylation motifs are coloured red, ubiquitinase-binding motifs are green, myristylation binding motifs are blue and glycosaminoglycan binding sites are pink. The presence of these motifs does not indicate that they are active. The degrees of conservation in NS3, the motifs and possible membrane arrangements are discussed in chapter 3.
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10 References


