Culicoides Fauna and Bluetongue Virus Serotype 8 Infection in South American Camelid Herds in Germany

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines **Dr. med. vet.**
beim Fachbereich Veterinärmedizin der Justus-Liebig-Universität Gießen

Claudia Schulz

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“One reason why research is so important is precisely that it can surprise you and tell you that your subjective convictions are wrong. If research always found what we expected, there wouldn't be much point in doing research.”

(Eugene T. Gendlin 1981)

"The most important thing is not to stop questioning."
(Albert Einstein 1955)

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CONTENTS

I. INTRODUCTION

Bluetongue (BT) is an infectious, non-contagious, notifiable disease caused by bluetongue virus (BTV), an *Orbivirus* in the *Reoviridae* family. Certain species of *Culicoides* biting midges are potential vectors of this virus infection (Mellor *et al*. 2000). BTV serotype 8 (BTV-8) was the first-ever serotype detected in Northern Europe. From 2006 to 2010, the BTV-8 epizootic in Europe (EU-BTNET system 2012) had substantial impact on animal welfare and probably caused greater economic damage than any previous single-serotype outbreak (Conraths *et al*. 2009, Wilson and Mellor 2009, Gethmann *et al*. 2010). BTV primarily causes severe disease in sheep, while other ruminant species and camelids usually exhibit subclinical disease. BTV-8, however, also affected cattle, several wild ruminant species and an alpaca. The manifestation of BT depends on virus strain, host species as well as breed, age, sex, individual resistance and fitness of the mammalian host (Ward *et al*. 1994, Brodie *et al*. 1998, Darpel *et al*. 2007, MacLachlan *et al*. 2009, Falconi *et al*. 2011).

BT monitoring and surveillance programmes have to include serological, virological and entomological surveillance in Member States within and outside of restricted zones (Commission regulation (EC) No 1266/2007, Caporale and Giovannini 2010). Although South American camelids (SAC) are susceptible to BTV, these species have not been included in the monitoring programmes. Before 2007, SAC were considered resistant to BT disease and the pathogenesis of BTV infection had so far not been investigated in these species (Rivera *et al*. 1987, Mattson 1994). However, fatalities related to BTV infection were reported in a few SAC during the recent BTV-8 and BTV-1 epizootics in Germany and France (Henrich *et al*. 2007, Meyer *et al*. 2009). This raised concern about their role in the epidemiology of BTV. To evaluate whether SAC are potential BTV reservoirs and a risk for the ruminant population, studies of BTV infection in SAC are required. The investigation of pathogenesis, diagnosis and frequency of BTV-8 infection in SAC as well as abundance of *Culicoides* vector species on SAC farms have been part of this work.

From 2008 to 2010, mandatory vaccination was successfully implemented in domestic ruminants to control the further spread of BTV-8 in Europe (Wilson and Mellor 2009, Zientara *et al*. 2010). Vaccination of over 80% of a susceptible population is considered the most effective strategy to control BT (Giovannini *et al*. 2004b, 2004c, Commission regulation (EC) No 1266/2007). SAC were generally not included in the compulsory vaccination programmes in Europe, and veterinary authorities were confronted with the question whether SAC should be vaccinated against BTV-8 at all. However, no information was available on

immunogenicity and safety of BTV vaccines or the vaccine dose to be used in camelids. In the presented work, long-term progression and colostral transfer of BTV antibodies as well as tolerance of BTV-8 vaccines were monitored after vaccination of SAC with inactivated BTV-8 vaccines.

II. LITERATURE REVIEW

1. Bluetongue virus

1.1. Taxonomy and virion structure of BTV

Bluetongue virus (BTV) is the prototype virus of the *Orbivirus* (*orbis* = lat. ring) species that belong to the *Reoviridae* family (Hewat *et al*. 1992, Verwoerd and Erasmus 2004). The orbiviruses BTV, *African horse sickness virus* (AHSV), *Epizootic hemorrhagic disease virus* (EHDV) and *Equine encephalosis virus* (EEV) can cause severe and economically important diseases of domestic and wild animals (Attoui *et al*. 2009). BTV as well as the phylogenetically closely related orbiviruses AHSV and EHDV are transmitted by the same *Culicoides* species (spp.) (Roy 1992, Hewat *et al*. 1992, Verwoerd and Erasmus 2004, Purse *et al*. 2005, Kampen and Kiel 2006, Meiswinkel *et al*. 2007, Mertens and Attoui 2009, Attoui *et al*. 2009).

Currently, BTV comprises 26 known serotypes (Fig. 1) (Mertens *et al*. 2004, Schwartz-Cornil *et al*. 2008, Hofmann *et al*. 2008, Maan *et al*. 2011a, Maan *et al*. 2011b, Maan *et al*. 2012).

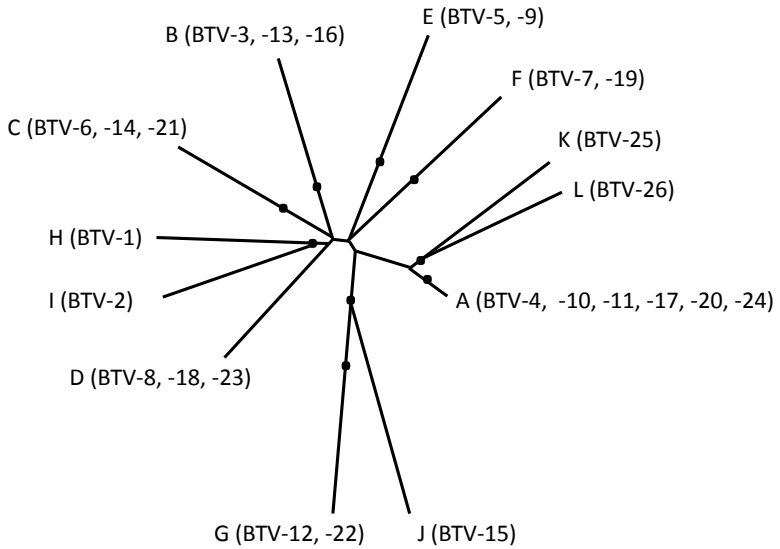


Fig. 1. Phylogenetic tree for Seg-2 (viral structural protein [VP]2 gene) sequences. This neighbour-joining tree shows serological relationships between reference strains of the current 26 BTV serotypes. The 10 evolutionary branching points are indicated by black dots on the tree, which correlate with the eleven Seg-2 'nucleotypes' designated A–L (figure source: Maan *et al*. 2011a, Maan *et al*. 2012, modified).

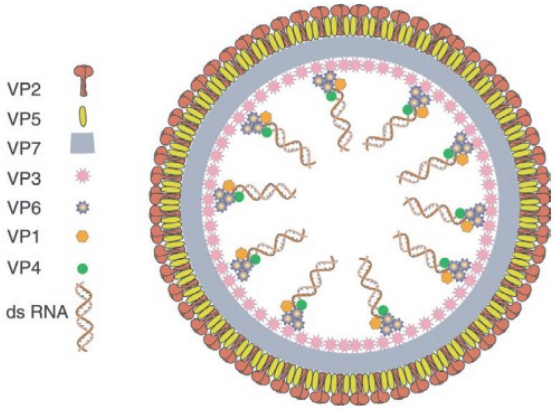


Fig. 2. Representative scheme of BTV structural proteins and double-stranded (ds)RNA segments. BTV particles contain three concentric layers of viral structural proteins (VP): the 'outer capsid' (VP2 and VP5), 'core' (VP7 and VP3) and the 'subcore' comprising the 'flower shaped' transcription complex (VP1, VP4 and VP6) and double-stranded (ds)RNA (figure source: Schwartz-Cornil *et al*. 2008, Mertens and Attoui 2009).

BTV is a non-enveloped virus with ten segments of double-stranded (ds)RNA packed in a triple-layered icosahedral protein capsid ('outer capsid, 'core' and 'subcore') of approximately 85 nm in diameter (Mertens *et al*. 2004, Schwartz-Cornil *et al*. 2008). For the virion structure of BTV see Fig. 2. The location, properties and functions of the viral structural proteins (VP1-7) and non-structural proteins (NS1-4 and 3a) of BTV are summarised in Table 1 in Appendix 1.

The outer capsid VP2 is the most variable protein and elicits serotype-specific neutralising antibodies and protective immunity (Verwoerd and Erasmus 2004, Noad and Roy 2009). In contrast, the second outer capsid VP5 is significantly more conserved, reflects the geographic origin and is a co-determinant of serotype (Schwartz-Cornil *et al*. 2008, Mertens and Attoui 2009). The core comprises two conserved major (VP7 and VP3) and three minor proteins (VP6, VP1 and VP4). The major proteins are group-specific and determine the morphological structure of the virus particle, while the minor proteins are responsible for the transcription of single-stranded messenger sense (m)RNA (Schwartz-Cornil *et al*. 2008). The capsomer VP7 forms the surface layer of the orbivirus core particle and is the immunodominant group-specific antigen. The considerable homology between S7 (VP7) of BTV, AHDV and EHDV has been explained by its function to bind to membrane proteins of the *Culicoides* vector (Verwoerd and Erasmus 2004). Cores are at least 100-fold more infectious for *Culicoides* midges or a *Culicoides* cell line (KC-cells, derived from *Culicoides* [*C.*] *sonorensis* embryos) compared to mammalian cells (Schwartz-Cornil *et al*. 2008, Batten *et al*. 2011). A possible

explanation has been an evolutionary adaptation to mammalian cells acquiring the outer capsid ring (Ross- Smith *et al*. 2009a).

The significant variations in the pathogenicity of BTV strains within a serotype (Schwartz-Cornil *et al*. 2008, Saegerman *et al*. 2008) are based on genetic differences that still remain uncharacterised (MacLachlan and Guthrie 2010). Comparison of strains of BTV from two regions showed that they could readily be distinguished by sequence analysis of their genome segment (S)10 (NS3/3A), but not their S2 (serotype-specific VP2) genes, which explains the diversification of BTV into distinct topotypes worldwide (Bonneau and MacLachlan 2004). In general, eastern and western topotypes can be distinguished, which are derived from different geographical areas (Schwartz-Cornil *et al*. 2008, Saegerman *et al*. 2008). The high genetic heterogeneity of BTV field strains is a consequence of genetic drift and, particularly, shift (Bonneau and MacLachlan 2004, He *et al*. 2010, Carpi *et al*. 2010).

1.2. Virus replication

The replication cycle of BTV was reviewed in detail by several authors (Roy 1992, Mertens *et al*. 2004, Schwartz-Cornil *et al*. 2008, Mertens and Attoui 2009, Noad and Roy 2009, Belhouchet *et al*. 2011, Ratinier *et al*. 2011). Briefly, after binding to the cell surface receptors via VP2 of the outer capsid, the BTV particle is internalised in the cell by receptor-mediated endocytosis (the core particle can also bind to insect cells via VP7, see II.1.1). The 'uncoated' core is released into the cytoplasm of the host cell. Subsequently, mRNAs are produced in the core by transcription complexes (VP1, VP4 and VP6). RNA synthesis takes place within the core to avoid recognition of RNA by the host cell, which would initialize antiviral defence mechanisms including apoptosis, interferon (IFN) production and RNA silencing. Recently, a fifth NS protein (NS4) has been detected (Belhouchet *et al*. 2011, Ratinier *et al*. 2011), which contributes to counteract the antiviral response of the host, but is dispensable for replication. Interestingly, although BTV replicates exclusively in the cytoplasm, recent investigations revealed that NS4 is also localized in nucleoli (Ratinier *et al*. 2011).

Synthesised mRNA copies are extruded in the cytoplasm for protein synthesis. Synthesis of negative-strand RNA and the assembly of dsRNA and progeny core particles takes place within the viral inclusion body (VIB) formed by NS2. The outer capsid proteins VP2 and VP5 are added as the core leaves the VIB. Mature virions are released by budding (mediated by NS3/NS3A), by direct cell membrane penetration (NS3) or (consequently) cell lyses. In contrast, BTV particles are released by budding in *Culicoides* cells and are therefore not

causing cell lysis. Released and enveloped virions lose the membrane and subsequently re-infect the same or other cells.

1.3. History, distribution and economic consequences

BT has been included in the list of notifiable diseases (formerly List A) of the OIE (Office International des Epizooties; syn. World Organisation for Animal Health) (MacLachlan 2011) as it is one of the most important diseases of domestic livestock worldwide causing substantial impact on economy and animal welfare (Mellor and Wittmann 2002, Saegerman *et al*. 2007, Gethmann *et al*. 2010). The disease was first recognised in South Africa and has probably occurred in this area for about one hundred years before it was described for the first time in the late 19th century by Hutcheon in 1880, particularly in imported (merino) sheep (Hateley 2009, MacLachlan and Guthrie 2010, MacLachlan 2011). Indigenous domestic and wild ruminants are usually highly resistant to clinical BT disease (Mellor and Wittmann 2002, Dal Pozzo *et al*. 2009b). Historically, African antelopes were probably the primary host species in the epidemiological cycle with *Culicoides* midges (Erasmus 1990). Today, the role of wild game has been taken over largely by cattle due to extensive farming (Erasmus 1990).

Over the last 60 years, the distribution of BTV has changed significantly, and virus isolations have successfully been conducted on every continent except Antarctica (MacLachlan and Guthrie 2010, MacLachlan 2011).

BTV is usually actively spread short distances by flying *Culicoides* or is disseminated (semi-) passively by *Culicoides* on prevailing winds over long distances (Purse *et al*. 2005, Burgin *et al*. 2012, Sedda *et al*. 2012). Other possible introduction routes of BTV in a region are: Movement of viraemic animals, transport of animal products (semen, embryos) or infected *Culicoides* vectors (Saegerman *et al*. 2008, Mintiens *et al*. 2008, Sedda *et al*. 2012).

Nowadays, enhanced global networking between countries and continents facilitates the incursion of new viruses and vectors in BTV-free areas and in naïve ruminant populations. Consequently, the risk for an encounter between serotypes and the possibility of reassortment have increased (Saegerman *et al*. 2008, Perry *et al*. 2011). Interestingly, BTV only remains enzootic for many years in regions where several serotypes are circulating. In contrast, a single virus serotype typically only occurs transiently in a region as for example BTV-8 in northern and central Europe (Gethmann *et al*. 2010, MacLachlan 2011, LAVES 2012).

1.3.1. BTV in Europe

Prior to 1998, BTV was considered an exotic disease in Europe. Only brief periodic incursions of the disease occurred in Southern Europe (Purse *et al*. 2005, Saegerman *et al*. 2008, Hendrickx 2009). The disease was restricted to parts of the world between latitudes of approximately 40 to 50° N and 35° S where *Culicoides* vector species occurred (MacLachlan and Guthrie 2010). In the following 7 years, five serotypes (BTV-1, -2, -4, -9 and -16) belonging to six strains have almost simultaneously invaded at least 12 countries in Europe from two or more directions. These invasions caused the most severe outbreak of BT on record (Purse *et al*. 2005).

The occurrence of BT in northern regions where *C. imicola* is rare or absent indicated that unknown vectors might play a role in the transmission of this virus (Mellor and Wittmann 2002, Saegerman *et al*. 2008). BTV isolation from indigenous Palearctic species belonging to the extremely abundant *C. obsoletus* and *C. pulicaris* complexes (Purse *et al*. 2005, De Liberato *et al*. 2005, Carpenter *et al*. 2009) dramatically expanded the risk of BTV transmission to whole Europe.

Period 2006-2012

BTV-8

Before 2006, the spread of BTV was mainly associated with the distribution of the tropical midge *C. imicola* forming an invisible border for BTV emergence to northern temperate regions in Europe. Therefore, the incursion of BTV-8 to north(-western) Europe was totally unexpected. This serotype was the first-ever BTV detected latitude >51°N, about 900 km further north than the previous European BTV incursions (Mellor and Wittmann 2002, Carpenter *et al*. 2009, Hendrickx 2009). BTV-8 was first detected in the Netherlands in August 2006 and subsequently in the surrounding countries Belgium, Germany, Luxemburg and France until the end of the year (Saegerman *et al*. 2008, Conraths *et al*. 2009, Mellor *et al*. 2009a, Gethmann *et al*. 2010).

Retrospective studies suggested that BTV has already been introduced during spring 2006, near to the National Park of Hautes Fagnes and Eifel in Belgium when *Culicoides* became active (Szmaragd *et al*. 2010, Saegerman *et al*. 2010). However, many other routes of introduction were possible (Mintiens *et al*. 2008). BTV-8 probably originated from sub-Sahara Africa. However, only a small number of reference strains were available, and sequence analyses did not point to a particular origin or introduction route. Consequently, the origin remains unknown (Maan *et al*. 2008, Mintiens *et al*. 2008, Carpenter *et al*. 2009). The

isolation of infectious BTV from non-engorged parous *C. obsoletus* confirmed earlier findings elsewhere, showing that species of this complex are potential BTV vectors (Saegerman *et al*. 2008).

Due to insufficient entomological, virological and serological surveillance data (Mellor *et al*. 2009c) and the unavailability of inactivated vaccines before spring 2008, the implementation of appropriate control measures was complicated (Carpenter *et al*. 2009, Caporale and Giovannini 2010). Imposed movement restrictions and administration of insecticides were not efficiently enough to contain the epizootic (Mehlhorn *et al*. 2008, Schmahl *et al*. 2009, Caporale and Giovannini 2010, Oura 2011). In contrast to previous experiences with the exophilic and exophagic main Afro-Asian vector *C. imicola*, stabling did not protect livestock against bites of the endophilic and endophagic Palearctic BTV vectors but even facilitated BTV transmission (Clausen *et al*. 2009).

The first BTV-8 case in Germany in 2008 was already detected in February, before the mandatory vaccination programmes in Europe were started (Hoffmann *et al*. 2008). Consequently, a high even though reduced number of outbreaks reoccurred in Germany in 2008. Furthermore, a dramatic increase of outbreaks and wide dissemination of BTV occurred in Europe in 2007 (Carpenter *et al*. 2009, Conraths *et al*. 2009, Caporale and Giovannini 2010, Oura 2011). Since then, the infection has spread from northwestern Europe as far as the Iberian Peninsula in the South, Scandinavia in the North and Israel in the East. Fig. 3 shows the restriction zones in Europe in January 2009, after the peak of BTV outbreaks (European Commission 2010, EU-BTNET system 2010b).

Approximately 89,000 BTV-8 outbreaks were registered in Europe from July 2006 to October 2010 (ADNS: EU-BTNET system 2008, 2009, 2010b). Due to its emergence in a region with ruminants that were naïve to any BTV serotype, this epizootic had devastating consequences on economy and animal welfare, probably causing greater economic damage than any previous single-serotype outbreak (Wilson and Mellor 2009). After the beginning of the compulsory vaccination campaigns in 2008, the number of cases decreased tremendously in the same year (~75% in Germany). However, owing to the late start of the vaccination regimens, the epizootic did not abate until 2009 (Conraths *et al*. 2009, Oura 2011).

In Germany, the last cases of BTV-8 infection were recorded in 2009. In whole Europe throughout 2010, circulation of BTV-8 was only reported in southern Spain and Italy (Oura 2011). As of 12 June 2010, Great Britain's BTV-8 status was changed from a protection zone to a lower risk zone (Gibbens 2010), and Britain was officially declared free from Bluetongue in July 2011 (DEFRA 2012). On 15 February 2012, Germany and the Benelux countries were

declared free of BTV-8 (LAVES 2012). The restricted zones in northern and central European countries were subsequently lifted as of March 2012 (European Commission 2012). However, a re-emergence of BTV-8 and the introduction of other serotypes or (*Culicoides* transmitted) arboviruses such as AHDV or EHDV have been considered likely (Carpenter *et al*. 2009, Hateley 2009, Eschbaumer *et al*. 2010a).

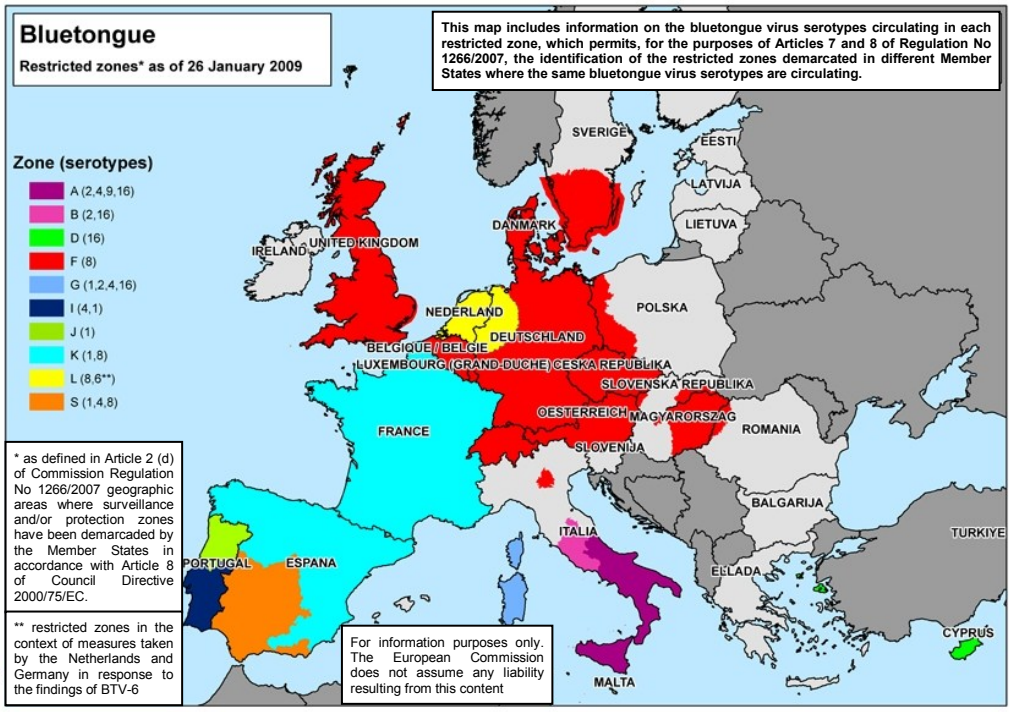


Fig. 3. Official veterinary BTV restriction zones in the European Union for all serotypes active on 26 January 2009 (figure source: http://ec.europa.eu/food/animal/diseases/controlmeasures/bluetongue_en.htm, modified).

BTV-1, -6, -11 and -25 in the episystem of BTV-8

In recent years, BTV-1 has expanded across the Iberian Peninsula, France, Sardinia and Greece (European Commission 2010, EU-BTNET system 2010a), while BTV-8 was spreading southwards. Since BTV-1 has crossed the *C. imicola* barrier in 2008, Mediterranean and temperate invasion zones of *C. imicola* and northerly *C. obsoletus* and *C. pulicaris* have largely overlapped, extending the areas of risk for BTV transmission (Purse *et al*. 2005, Hendrickx 2009, Carpenter *et al*. 2009, Hateley 2009).

Toggenburg orbivirus (TOV) was found in goats in Switzerland in early 2008, but did not spread to other European countries (Hofmann *et al*. 2008, Maan *et al*. 2011a). In late 2008, BTV-6 was detected in the Netherlands, northwestern Germany and Belgium (Fig. 3), and several cases of BTV-11 were detected in Belgium. Both serotypes did not return in 2009

although no control measures were implemented (Hateley 2009, Eschbaumer *et al*. 2010a, van Rijn *et al*. 2012).

1.3.2. Transmission and overwintering mechanisms of BTV

BTV is almost exclusively transmitted by certain *Culicoides* spp. In general, BTV transmission by contact or by animal products such as meat or milk does not occur (Papadopoulos *et al*. 2009). However, other routes of transmission are possible. Ticks and *Melophagus ovinus* were suggested as mechanical vectors (Wilson *et al*. 2008, Bouwknegt *et al*. 2010). Transplacental transmission was repeatedly reported during the BTV-8 epizootic in Europe (Menzies *et al*. 2008, De Clercq *et al*. 2008a, De Clercq *et al*. 2008b, Backx *et al*. 2009, Darpel *et al*. 2009a, Williamson *et al*. 2010, van der Sluijs *et al*. 2011). In the past, transplacental infection of BTV was associated with laboratory-adapted BTV strains, in particular live attenuated vaccine virus strains, but not with wild-type viruses. Recently, transplacental transmission to a llama was suggested for a wild-type BTV-1 for the first time (Meyer *et al*. 2009, EFSA Report 2011). The detection and isolation of BTV from calves born from a BTV seropositive but BTV RNA negative cow demonstrated that pregnant dams that were infected during gestation pose a risk when transported to BT-free regions. Therefore, more severe trade restrictions for pregnant dams are required (see II.5) (De Clercq *et al*. 2008a). Regarding the significance of BTV shedding in semen, opinions are contradictory (Wrathall *et al*. 2006, Napp *et al*. 2011).

Possible overwintering mechanisms of BTV include the transmission paths already mentioned in this section. Purse *et al.* (2005) provided an overview of possible overwintering mechanisms of BTV. Whether transovarial transmission is possible in the vector is still under debate (Mellor *et al*. 2000, White *et al*. 2005, Purse *et al*. 2005).

The prolonged viraemia characteristic for BTV infection in ruminants facilitates the transmission of BTV to *Culicoides* after the cold winter months when no or only a few adult *Culicoides* are active. Unlike former opinions, a vector-free period does generally not exist in temperate regions, especially in warm winters with a short cold period such as the first winter after the emergence of BTV-8 to Northern Europe (2006/2007) (Wilson *et al*. 2008). Warmer temperatures in stables additionally increase and protract the risk of BTV transmission during cold weather conditions.

A possible role of the spleen for BTV persistence in ruminants was ruled out as virus isolation and immunofluorescence labelling, which indicate virus replication, were markedly reduced or failed (Mahrt and Osburn 1986, reviewed by MacLachlan *et al*. 2009, Darpel *et al*. 2009b,

Worwa *et al*. 2010, Darpel *et al*. 2012). Vascular endothelial cells (ECs) and agranular leukocytes in the skin were suggested as a major source of BTV infection for *Culicoides* (Darpel *et al*. 2012). Therefore, persistent infection of γδ T-cells in the skin could be an efficient overwintering mechanism, but has so far not been confirmed (Takamatsu *et al*. 2003, Purse *et al*. 2005, Lunt *et al*. 2006, Wilson *et al*. 2008, López-Olvera *et al*. 2010, Darpel *et al*. 2012).

2. *Culicoides* biting midges

2.1. Taxonomy and morphology of ceratopogonids

Ceratopogonidae comprise some 125 genera with about 5,500 species (Mellor *et al*. 2000). In Germany, four subfamilies (Palpomyiinae, Ceratopogoninae, Dasyheleinae and Forcipomyiinae) with 332 ceratopogonid species were identified by Havelka and Aguilar (1999; cited by Werner and Kampen 2007). *Culicoides* are 0.5 to 3 mm small biting midges that belong to the family Ceratopogonidae (Diptera) (Mellor *et al*. 2000, Werner and Kampen 2007). Over 1,400 species belonging to the genus *Culicoides* (subfamily Ceratopogoninae) are known to occur worldwide, except in the extreme polar regions, New Zealand, Patagonia and the Hawaiian Islands (Mellor *et al*. 2000, Borkent 2012).

Ceratopogonids can be identified by their piercing-sucking mandibles, veining of the (often hairy) wings, genitalia, shaping of legs and habitat preferences. Males can usually be differentiated from females by their bushy antennae (Werner and Kampen 2007, Mehlhorn *et al*. 2009c). Species are morphologically differentiated from each other by wing patterns. For further details on morphological characteristics, see more specialist literature (Werner and Kampen 2007). Wings of females of sibling/complex species look very similar or cannot be morphologically discriminated from each other. Two species complexes of Palearctic *Culicoides* predominantly occur in Germany (Hoffmann *et al*. 2009a) and in some southern and eastern European countries (Purse *et al*. 2005): The *C. obsoletus sensu lato* (Obsoletus complex) and *C. pulicaris s.l.* (Pulicaris complex) comprising three (*C. obsoletus sensu stricto*, *C. scoticus*, *C. chiopterus*) and six (*C.pulicaris s.s.*, *C. punctatus*, *C. impunctatus*, *C. lupicaris/delta*, *C. fagineus*, *C. newsteadi*) species, respectively. Fig. 4 and Fig. 5 show the characteristic wing patterns of those two complexes. Species of both complexes have been incriminated as vectors of BTV (Hoffmann *et al*. 2009a). Occasionally, *C. dewulfi*, a novel potential vector of BTV, was considered part of the Obsoletus complex, but phylogenetic analysis suggested that this species is phylogenetically close to *C. imicola* and should be

Fig. 4. *Culicoides obsoletus* female, a potential vector of BTV, engorged with fresh blood.

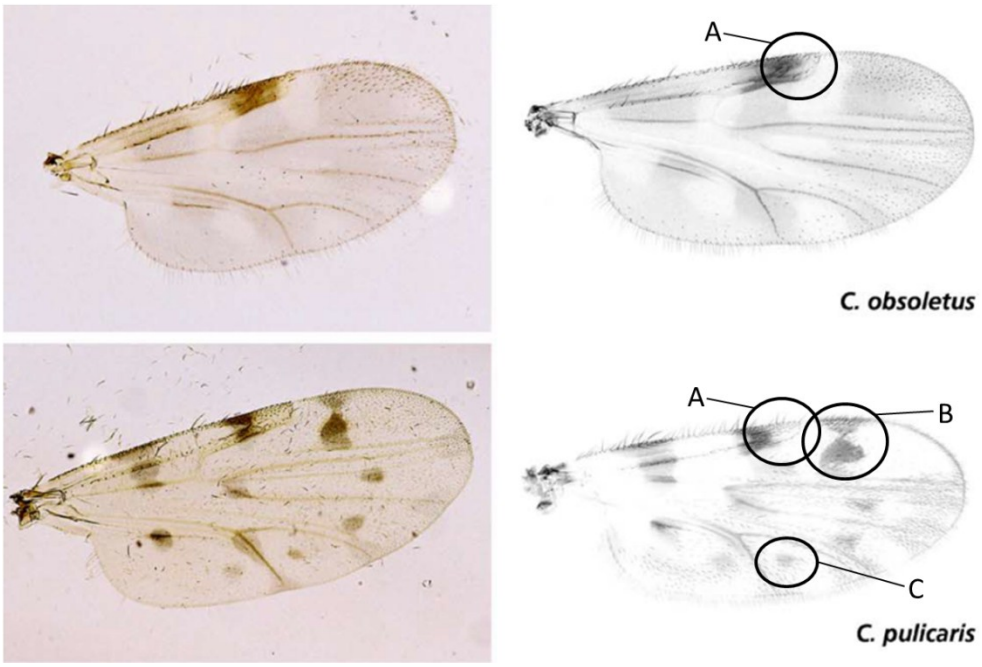


Fig. 5. *Culicoides* wings. Light micrographs (left side) and diagrammatic representations of the wings of *C. obsoletus* and *C. pulicaris* (figure source: Mehlhorn *et al*. 2007, modified). In both complexes the second radial cell of the wing is partly or completely pale (A). *C. obsoletus s.l.* has wings with indistinct pale markings or pale areas, while species belonging to the Pulicaris complex present wings with distinct dark markings on a pale ground, an hourglass-shaped marking after the costa (B) and a dark spot in the cubital cell (C) (www.culicoides.net).

treated as a separate taxonomic group (Dewulfi complex) (Meiswinkel *et al*. 2004, Schwenkenbecher *et al*. 2009a). The major Old World vector of BTV, the *C. imicola* species complex, is currently represented by 10 species (Sebastiani *et al*. 2001, Purse *et al*. 2005). This complex mainly occurs in Africa, Asia and also in southern Europe, but has so far not

been detected north of the Alps (Purse *et al*. 2005, Kaufmann *et al*. 2009, Hoffmann *et al*. 2009a).

To identify females to species level, cytotaxonomic, biochemical or molecular biological methods are used (Werner and Kampen 2007) (see also II.2.4). In contrast, males can be determined to species by inspection of their genitalia (Boorman 1986, Pili *et al*. 2010, Vilar *et al*. 2011).

2.2. Biology and breeding habitats of ceratopogonids

The development of ceratopogonids from egg to imago depends on temperature and takes at least three weeks in temperate regions, but can be prolonged to more than seven months during winter. See Fig. 6 for the life cycle (Kampen and Kiel 2006, Mehlhorn *et al*. 2007, Kampen *et al*. 2007). Similar to many other insect families, overwintering takes place in the fourth larval stage (Werner and Kampen 2007).

The four larval instars and the pupal stage require a certain amount of moisture to avoid desiccation and to facilitate fast development. For the same reason, adult *Culicoides* are mainly active from dusk until dawn when temperatures are lower and humidity is higher. The incidence of *Culicoides* can be affected by nearby surface water or marshland as *Culicoides* use this for breeding (Kampen and Kiel 2006, Kampen *et al*. 2007, Werner and Kampen 2007, Purse *et al*. 2012). Habitats and larval developmental substrates depend upon species and range widely, from the tropics to the tundra and from sea level up to 4000 m (Mellor *et al*. 2000, Mercer *et al*. 2003, Kaufmann *et al*. 2009, Tschuor *et al*. 2009, Foxi and Delrio 2010, Ninio *et al*. 2011). Sibling species are morphologically identical or very similar (see II.2.1). However, their behaviour, (breeding) habitat preferences and other biological characteristics can be very distinct (Werner and Kampen 2007, Mullen 2009).

Culicoides usually stay a few hundred meters around their breeding habitats, if not transported as aerial plankton over much greater distances by winds (Mellor 2001, Alba *et al*. 2004, Purse *et al*. 2005, Gloster *et al*. 2007, Hendrickx *et al*. 2008, Hendrickx 2009, García-Lastra *et al*. 2012). Since females of most (haematophagous) species (96%) need blood for egg production, habitats are regularly near vertebrate hosts (Mellor *et al*. 2000). For example, the taxons *C. dewulfi* and *C. chiopterus* breed and overwinter in cattle and horse dung, *C. obsoletus* and *C. scoticus* in maize silage residues (Meiswinkel *et al*. 2008, Balenghien *et al*. 2008, Zimmer *et al*. 2008).

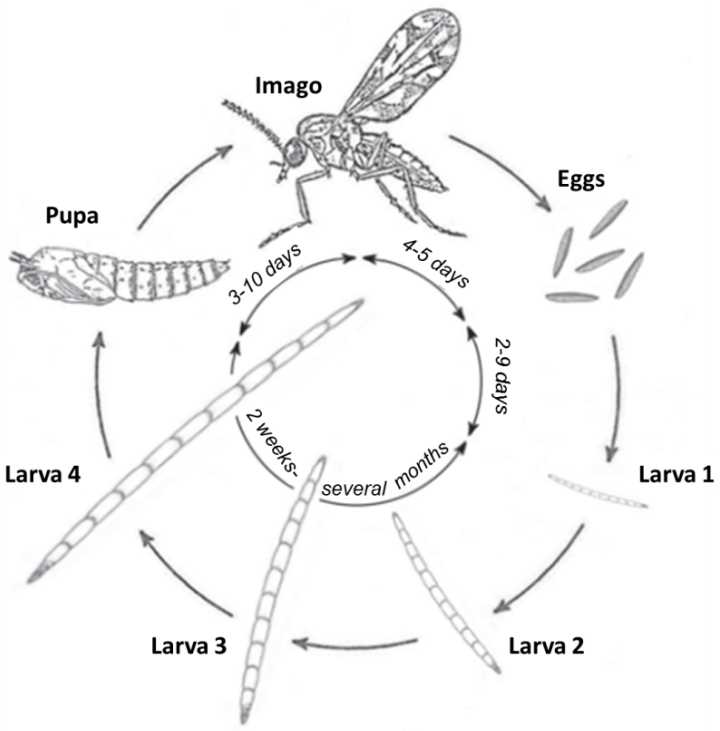


Fig. 6. Holometabolic life cycle of ceratopogonids (Kampen and Kiel 2006, modified).

2.3. Methods of ceratopogonid collection

Various methods have been used to collect biting midges (Mellor *et al*. 2000, Mercer *et al*. 2003, Balenghien *et al*. 2008). However, different trapping methods and monitoring systems can suggest different activity patterns, alter the sensitivity of detection of (infected) *Culicoides* and, therefore, complicate the comparison of results from different studies (Mellor *et al*. 2000, Meiswinkel *et al*. 2008, Mehlhorn *et al*. 2009a, Mehlhorn *et al*. 2009c, Mayo *et al*. 2012b). Good correspondence was found between light trap catches and emergence trends (Foxi and Delrio 2010). However, abundance, composition or BTV infection rates of larval instars respectively adult *Culicoides* collected in- and outdoor depend on the location (Clausen *et al*. 2009, Ninio *et al*. 2011).

The Onderstepoort-type black-light trap is considered the 'gold standard' (Meiswinkel *et al*. 2008). This trap showed the highest sensitivity in comparison to four other blacklight traps including the BG-Sentinel^{TM} UV light trap (Venter *et al*. 2009, Del Río *et al*. 2009), which was used for the entomological surveys in Germany 2007/2008 (Hoffmann *et al*. 2009a). However, while Venter *et al.* (2009) demonstrated significantly better results for the Onderstepoort trap, no significant differences could be demonstrated by Del Río *et al.* (2009).

2.4. Molecular identification of *Culicoides*

Morphological identification of *Culicoides* spp. is laborious using morphological keys, which mainly refer to wing patterns, reproductive organs or papal segments (see II.2.1). Therefore, various molecular methods have been developed to facilitate and speed up the accurate identification of *Culicoides* (Stephan *et al*. 2009). Genus-specific forward and reverse primers were designed to amplify internal transcribed spacer 1 and 2 (ITS-1 and ITS-2), which is located in the mitochondrial ribosomal DNA (rDNA) (see Fig. 7) (Gomulski *et al*. 2006, Stephan *et al*. 2009, Hoffmann *et al*. 2009a). Vanbinst *et al.* (2009) newly developed a real-time quantitative reverse transcription-PCR (RT-qPCR) for the detection of *Culicoides* using 18S rDNA as internal control. Various alternative molecular methods are described by other groups (Mathieu *et al*. 2007, Monaco *et al*. 2010, Kaufmann *et al*. 2012, Deblauwe *et al*. 2012, Wenk *et al*. 2012). Besides adult midges, molecular identification of *Culicoides* larvae advances the understanding of breeding habitats and, hence, the epidemiology of BTV (Stephan *et al*. 2009, Schwenkenbecher *et al*. 2009b).

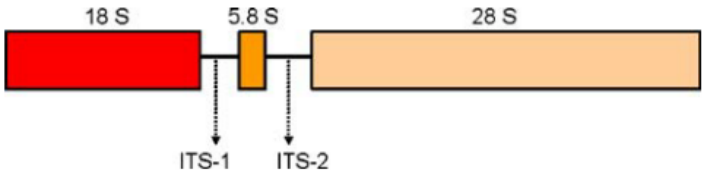


Fig. 7. Mitochondrial ribosomal DNA (rDNA) segment in *Culicoides*. Schematic illustration of the genetic regions located in rDNA that are important for the detection of *Culicoides* by PCR (figure source: Stephan *et al*. 2009). The rDNA contains highly conserved regions (5.8S, 18S and 28s rDNA genes) and the more variable (non-coding) regions ITS-1 and -2. Genus-specific primers bind to the conserved regions flanking the ITS-1 and -2 regions and ensure the amplification of the ITS regions (Gomulski *et al*. 2006, Stephan *et al*. 2009, Hoffmann *et al*. 2009a).

2.5. *Culicoides* as vectors and nuisance

Culicoides spp. belong to the world's smallest blood sucking insects (Mellor *et al*. 2000). Usually blood is required for their egg development and subsequent oviposition, as in other haematophagous insect females (Mullen 2009). In contrast, males only feed on plant-juice (Mehlhorn *et al*. 2007).

Many species of biting midges feed primarily on mammals (including humans), whereas others feed preferentially on birds, reptiles or amphibians (Mellor *et al*. 2000, Mullen 2009,

Lassen *et al*. 2012). Some species are quite host-specific, while others are considered generalists and feed on alternative hosts of different species or classes.

Culicoides are pool feeders as they lacerate the skin to feed on the accruing effusion including blood, lymph and skin cells (Darpel *et al*. 2012). Their bites can be very painful (Mullen 2009) and may cause strong allergic dermatitis in horses (known as 'sweet itch') (Heimann *et al*. 2011, Schaffartzik *et al*. 2011) and in ruminants (Connan and Lloyd 1988, Yeruham *et al*. 1993, Corrêa *et al*. 2007). The appearance of a huge number of *Culicoides* can also be a serious nuisance for humans (Santamaría *et al*. 2008, Logan *et al*. 2009).

Only about 50 species of the approximately 1,400 *Culicoides* spp. are potential vectors of various pathogens, including filaria and protozoa. However, they attain greatest importance as vectors of viruses. To date, over 60 viruses have been isolated worldwide (Mellor *et al*. 2000, Purse *et al*. 2005, Werner and Kampen 2010). Eight viruses belonging to the *Bunyaviridae*, *Rhabdoviridae* and *Reoviridae* families are important for ruminants (Stephan *et al*. 2009, Reeves 2010). Recently, a new *Orthobunyavirus*, "Schmallenberg virus" (SBV), has been discovered in ruminants and in the species *C. obsoletus* and *C. dewulfi* in northern Europe (Hoffmann *et al*. 2012, ECDC 2012, Rasmussen *et al*. 2012). Camelids are also susceptible to infection with SBV and other orthobunyaviruses (Jack *et al*. 2012, Schulz *et al*. 2012a).

BTV in *Culicoides* vectors

The distribution and seasonality of BT depends on the presence, activity and vector capacity of *Culicoides* (Losson *et al*. 2007, Gloster *et al*. 2007, Wilson *et al*. 2007, Baldet *et al*. 2008, Hoffmann *et al*. 2008, Hoffmann *et al*. 2009a). On a world-wide scale, the most important BTV vectors are *C. sonorensis* in North America, *C. insignis* in Central and South America, *C. imicola* and possibly *C. bolitinos* in Africa, *C. brevetarsis*, *C. fulvus*, *C. wadai*, *C. actoni* in Australia, *C. imicola* and the latter four species in Asia, and *C. imicola*, *C. obsoletus*, *C. pulicaris* in Europe, (Tabachnick 2004, Hoffmann *et al*. 2009a, Mellor *et al*. 2009b). *C. dewulfi* was found to be a potential new vector of BTV in northern Europe (Meiswinkel *et al*. 2007, Stephan *et al*. 2009).

BTV is not or very rarely transmitted during the winter months (Losson *et al*. 2007, Gloster *et al*. 2007, Wilson *et al*. 2007, Baldet *et al*. 2008, Hoffmann *et al*. 2008, Hoffmann *et al*. 2009a). According to recent studies, a vector-free period in winter does not exist in Germany (Fig. 8) and western adjacent countries, but may occur at certain locations, for example in central Germany (Meiswinkel *et al*. 2008, Clausen *et al*. 2009, Hoffmann *et al*. 2009a).

Due to the long-term viraemia characteristic for BTV infection in ruminants, a never ending transmission cycle is maintained between *Culicoides* vectors and susceptible ruminant hosts (Mellor 1990). BTV is promiscuous between many host and vector species (Purse *et al*. 2005) facilitating the spread of BT (Batten *et al*. 2011).

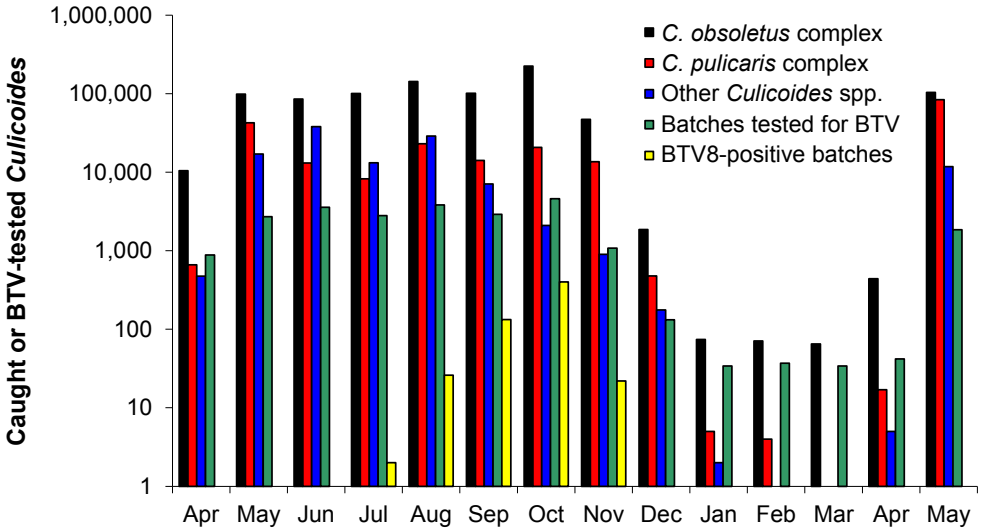


Fig. 8. *Culicoides* caught in Germany 2007/2008. Monthly catches of midges of the *C. obsoletus* complex, *C. pulicaris* complex and other *Culicoides* spp. captured with 89 black-light traps in Germany during 7 consecutive nights in the first week of each month during the study period (April 2007–May 2008). Batches consisting of <50 female biting midges were tested for BTV by RT-qPCR. The total number of batches (green) and the number of batches positive for BTV (yellow) are shown. From June to November 2007, a total of 585 (n=1 to 401) *Culicoides* were tested positive for BTV (Hoffmann *et al*. 2009a; Figure courtesy of F. J. Conraths, FLI, Wusterhausen, modified).

Factors influencing *Culicoides* abundance and BTV infection rates

Complex local interacting factors influence *Culicoides* abundance, breeding sites, vector-competence and BTV infection rates in ruminants. These include anthropogenic, landscape, biotic (forest cover and vegetation activity), abiotic (topography, temperature and aridity) and meteorological factors (Tabachnick 2004, Mullens *et al*. 2004, Purse *et al*. 2005, Conte *et al*. 2007, Staubach *et al*. 2007, Hörbrand and Geier 2009, Vorsprach *et al*. 2009, De Liberato *et al*. 2010, Purse *et al*. 2012, Mayo *et al*. 2012a). Durand *et al.* (2010a) suggested that surrounding natural ecosystems have played a substantial role in the circulation of BTV-8 in France. Local density of cattle had a protective effect on the risk of BTV infection since cattle was found to be the most attractive host for Palearctic biting midges (Bartsch *et al*. 2009, Durand *et al*. 2010a), like horses (Viennet *et al*. 2012). Furthermore, breeding sites of BTV

vectors in northern Europe were found mainly in anthropogenic environments, close to farms (Zimmer *et al*. 2008, Meiswinkel *et al*. 2008).

Activity and number of *Culicoides* are positively correlated with temperature (Mellor *et al*. 2000, Kiel *et al*. 2009, Vorsprach *et al*. 2009). Adult *Culicoides* are short-lived. Most individuals probably survive for about 10 to 20 days and during this time may take multiple blood meals (Mellor *et al*. 2000). Exceptionally they may live for much longer periods. At temperatures below about 10°C, the activity of *Culicoides* is generally suppressed (Mellor *et al*. 2000).

BTV transmission becomes possible after the extrinsic incubation period (EIP), which is the interval between ingestion of BTV and its release in the saliva (Mellor *et al*. 2009b). After this period, vectors usually remain infected for life (Mellor 1990). A single bite of an infected culicoid midge suffices for BTV transmission (Baylis *et al*. 2009). The successful completion and time of the EIP is influenced by several internal and external factors (Mellor *et al*. 2009b) and is restricted to a relatively small number of species (Mellor 1990). *C. obsoletus/scoticus* occurs at comparable low temperatures (Kampen *et al*. 2007) and was caught all year round at some locations in Germany, independently from their position above sea level (Hoffmann *et al*. 2009a, Mehlhorn *et al*. 2009c). Nevertheless, the number of *C. obsoletus* correlated with temperature, which could explain their significantly lower abundance at higher places (Mehlhorn *et al*. 2009c). *C. pulicaris* incidence depends on a high, stable relative humidity, prevails with increasing altitude, and shows a bimodal seasonal dynamic (maximum reached in spring and autumn) in some regions (Kampen *et al*. 2007, Kaufmann *et al*. 2009, Tschuor *et al*. 2009). In Europe, seasonal activity and number of peaks vary and can be caused by different generations (Boorman 1986, Vorsprach *et al*. 2009).

Virus transmission and replication in BTV vectors (vector competence) is crucially affected by temperature (Wittmann *et al*. 2002, Paweska *et al*. 2002, Carpenter *et al*. 2011). The minimum temperature for BTV replication in vectors lies between approximately 10 and 15 °C, while at a temperature of 25 °C transmission becomes possible at 7 to 15 days post infection (dpi) (Mellor *et al*. 2000, Kampen *et al*. 2007, Mullen 2009, Mellor *et al*. 2009b). Interestingly, the gut wall of the non-vector species *C. nubeculosus* got permeable for BTV when it was exposed to a temperature of 33°C (Mellor *et al*. 2000, Mellor *et al*. 2009b). Thus, the unusually warm weather conditions in Europe may have caused an increase in vector-competence of *C. obsoletus* and *C. pulicaris* (Mellor *et al*. 2000, Mellor *et al*. 2009b). A marked increase of BT incidence was found in regions in Europe where climate warming was greatest (Purse *et al*. 2005).

3. BTV in the mammalian host

3.1. Pathogenesis of BTV infection in ruminants

Pathogenesis and clinical signs are generally similar in ruminants infected with virulent BTV strains and have been thoroughly reviewed by several authors (Verwoerd and Erasmus 2004, MacLachlan *et al*. 2009, MacLachlan and Gard 2009, Darpel *et al*. 2009b, Eschbaumer *et al*. 2010a, MacLachlan 2011).

After inoculation of BTV in ruminant skin with saliva by a single bite of a *Culicoides* midge (Wilson and Mellor 2008, Baylis *et al*. 2009), BTV primarily targets conventional dendritic cells in skin lymph, which are contributing to the primary dissemination of BTV from the skin to draining lymph nodes (Hemati *et al*. 2009). Early replication of BTV simultaneously (≤ 2-3 dpi) takes place in regional capillary ECs of the skin and in agranular mononuclear leukocytes (peripheral blood mononuclear cells, PBMC) of lymphatic organs (Darpel *et al*. 2012). IFN-α is induced by BTV and plays an essential role in the antiviral innate immune response leading to the decrease after the first viraemic peak (Foster *et al*. 1991, Calvo-Pinilla *et al*. 2009a).

The second and higher viraemic peak was previously explained by massive secondary replication in PBMC and ECs of several organs resulting in a generalised viraemia (MacLachlan *et al*. 1990, Barratt-Boyes and MacLachlan 1994). However, tissue tropism and "organ-manifestation" were recently suggested to explain virus replication in skin, lymph nodes of the head, tonsils and labia in the early stage of infection. In contrast, ECs of less susceptible organs (for example heart, muscle and liver) may be infected when viraemia reaches a high level (Darpel *et al*. 2012). The reduction of the second peak is probably due to a combination of IFN activity and seroconversion together with T-cell mediated immune response (Foster *et al*. 1991, Channappanavar *et al*. 2012).

Since *Culicoides* lacerate the skin to feed from a blood pool mixed with skin cells and lymph, they may already be infected 2-3 dpi when systemic viraemia is still low in the mammalian host.

BTV is highly cell-associated and replicates in a wide range of cell types and at various temperatures, facilitating infection and replication in both the *Culicoides* and the mammalian host (Wilson *et al*. 2007, MacLachlan *et al*. 2009, Darpel *et al*. 2009b, Sánchez-Cordón *et al*. 2010). In contrast, BTV does not replicate in red blood cells (RBC) but persists within invaginations of the cell membranes throughout viraemia. This mechanism protects the adsorbed virions from immune clearance (Barratt-Boyes and MacLachlan 1994, Brewer and MacLachlan 1994). Prolonged viraemia and co-circulation with neutralising antibodies is

characteristic for BTV infection in ruminants. The duration of viraemia corresponds to the lifespan of RBC (Brewer and MacLachlan 1994, Katz *et al*. 1994, Bonneau *et al*. 2002). Infectious virus can be isolated up to about 60 dpi (occasionally up to 100 dpi), while BTV RNA was detected about 160 up to 222 dpi using RT-PCR (Katz *et al*. 1994, Barratt-Boyes and MacLachlan 1994, Bonneau *et al*. 2002, Mertens *et al*. 2009). Adsorption of the virus to RBC over several weeks increases the likelihood of BTV infection of haematophagous *Culicoides* vectors and facilitates onward transmission (Barratt-Boyes and MacLachlan 1994, MacLachlan *et al*. 2009). In general, the time of a possible BTV infection of *Culicoides* is in accordance with the duration of viraemia - when infectious virus is detectable - in ruminant blood (Bonneau *et al*. 2002).

BTV induces apoptosis and/or necrosis in mammalian cell lines, microvascular ECs and monocytes, but not in insect cells, γδ T-cells and activated blood lymphocytes (DeMaula *et al*. 2001, Takamatsu *et al*. 2003, Mortola *et al*. 2004, Schwartz-Cornil *et al*. 2008, Drew *et al*. 2010a). The damage of the endothelial lining in small blood vessels results in haemorrhages and tissue infarction (thrombosis). In white-tailed deer, BTV occasionally causes consumptive coagulopathy (Vosdingh *et al*. 1968, Howerth *et al*. 1988, DeMaula *et al*. 2002, Verwoerd and Erasmus 2004, MacLachlan *et al*. 2009, MacLachlan and Gard 2009). Additionally, activation of ECs and macrophages induces the production of vasoactive mediators, which can promote cell death or cause vascular permeability with subsequent oedema, in particularly pulmonary oedema (DeMaula *et al*. 2001, MacLachlan *et al*. 2009, Mortola and Larsen 2010, Drew *et al*. 2010b, Sánchez-Cordón *et al*. 2012). BTV-infected monocytes and lymphocytes also respond with inflammatory and antiviral responses (MacLachlan and Gard 2009, Ross- Smith *et al*. 2009b). Lymphopenia, a common sign of BT, possibly occurs due to destruction or sequestration of lymphocytes at virus replication sites (Darpel *et al*. 2009b).

Neutralising antibodies (NAbs) recognise epitopes of the serotype-specific outer capsid proteins and, therefore, protect against challenge with the homologous serotype. However, other factors contribute to the protection against BTV re-infection. Cross-protection against heterologous serotypes is mediated by cytotoxic T-cells (CTL), which recognise intracellular antigens of the highly conserved NSs and viral core proteins via the major histocompatibility complex (MHC) pathway (Jeggo *et al*. 1984, Darpel *et al*. 2009b, Sánchez-Cordón *et al*. 2010, Hund *et al*. 2012). Therefore, ruminants that have previously been infected or immunised can be protected against BTV re-infection, even if they are negative for NAbs (Savini *et al*. 2004b, Eschbaumer *et al*. 2009, Savini *et al*. 2009, Oura *et al*. 2010).

3.2. Clinical signs and post-mortem lesions in ruminants

Host range

BT manifestation depends on species, breed, age, sex, individual resistance and fitness of the mammalian host and on virus strain (Sellers 1984, Ward *et al*. 1994, Brodie *et al*. 1998, Thiry *et al*. 2006, reviewed by MacLachlan *et al*. 2009, Savini *et al*. 2010, Linden *et al*. 2010, García-Bocanegra *et al*. 2011). The host range susceptible to BTV infection includes various domestic (sheep, goats and cattle) and wild ruminant species (deer, mouflon, ibex, yak, European and American bison and musk ox) as well as camelids (House *et al*. 1982, Erasmus 1990, Tessaro and Clavijo 2001, Mauroy *et al*. 2008, Ludwig and Silinski 2008, reviewed by MacLachlan *et al*. 2009, García *et al*. 2009). BTV primarily causes disease in sheep. In contrast, cattle, goats, various species of wild ruminants and camelids usually exhibit subclinical infection (Rivera *et al*. 1987, Mattson 1994, Darpel *et al*. 2007, Backx *et al*. 2007, MacLachlan *et al*. 2008, Ruiz-Fons *et al*. 2008, Fernandez-Pacheco *et al*. 2008, Mauroy *et al*. 2008, Wernery *et al*. 2008, Elbers *et al*. 2008b, MacLachlan *et al*. 2009, Dal Pozzo *et al*. 2009a, 2009b, López-Olvera *et al*. 2010, Linden *et al*. 2010, Batten *et al*. 2011). However, the pathogenesis of BTV infection is similar in sheep and cattle. Inherent species-specific differences in the production and activities of EC-derived mediators were incriminated to contribute to the sensitivity of sheep and deer to BTV-induced microvascular injury (DeMaula *et al*. 2001, MacLachlan and Gard 2009). Differences in the susceptibility of mammalian hosts to BT disease probably depend on the variability of toll-like receptor 3 (TLR3) expression in tissues (Vos *et al*. 2009). A significantly higher ratio of thromboxane to prostacyclin has been found in sheep indicating enhanced coagulation (disseminated intravascular coagulation, DIC) and a subsequent bleeding tendency (DeMaula *et al*. 2002, MacLachlan and Gard 2009).

BTV infection in non-artiodactyls was reported in domestic and wild carnivores and rhinoceros (Akita *et al*. 1994, Evermann *et al*. 1994, Osburn 1994, Alexander *et al*. 1994, Ianconescu *et al*. 1996, Fischer-Tenhagen *et al*. 2000, Jauniaux *et al*. 2008, Oura and El Harrak 2010). Carnivores may be infected by oral ingestion of infected meat or meat products or through vector feeding (Alexander *et al*. 1994, Oura and El Harrak 2010). To date, their role in the transmission cycle of BTV remains unknown (Oura and El Harrak 2010), but it is unlikely that carnivores play a significant role in the epidemiology of BTV (MacLachlan 2011).

Clinical signs

Clinical signs and lesions reflect virus-mediated vascular injury and subsequent immune and repair responses after BTV infection. None of the clinical signs are pathognomonic for BT (Mertens *et al*. 2009). Animals with acute BT can have any combination of signs (MacLachlan and Gard 2009), and secondary infection such as bacterial pneumonia may aggravate BT disease and subsequently promote fatality (MacLachlan and Gard 2009). Therefore, other diseases with similar clinical signs should be considered (Mertens *et al*. 2009). Suspicion of BTV infection and disease has to be confirmed by diagnostic test procedures (see II.4). A list of possible differential diagnoses was given by several authors (Verwoerd and Erasmus 2004, Bexiga *et al*. 2007, Williamson *et al*. 2008, Mertens *et al*. 2009, OIE 2011).

In contrast to previously known BTV strains, a remarkable high proportion of cattle showed overt clinical signs to infection with the European BTV-8, indicating an increased virulence of this strain (Thiry *et al*. 2006, Elbers *et al*. 2008a, Dal Pozzo *et al*. 2009a, reviewed by Dal Pozzo *et al*. 2009b). Nevertheless, clinical signs and lesions in sheep were much more prominent and different than in cattle (Elbers *et al*. 2008b). Clinical signs indicative for BTV-8 infection in affected sheep flocks were erosions of the oral mucosa, fever, salivation, facial and mandibular oedema, apathy and tiredness, oedema of the lips, lameness and dysphagia were among the clinical signs most frequently recorded. In contrast, the most prominent clinical signs in affected cattle herds included crusts/lesions of the nasal mucosa, erosions of lips/crusts in or around the nostrils, erosions of the oral mucosa, salivation, fever, conjunctivitis, coronitis, muscle necrosis and stiffness in limbs (Elbers *et al*. 2008b).

Besides, BTV-8 can cause abortion, congenital deformities and cerebral abnormalities leading to congenital neurological signs, dullness or weakness (Wouda *et al*. 2008, De Clercq *et al*. 2008a, Wouda *et al*. 2009). Congenital deformities in live-born calves mostly occurred after BTV-8 infection of dams in early gestation (Wouda *et al*. 2009). However, the highest transmission rate was found at mid-term gestation (69%) (van der Sluijs *et al*. 2011).

Post-mortem lesions

Lesions typically found in BTV-infected ruminants at post-mortem examination are consequences of the damages to the vascular system. These include widespread, but often localised, hyperaemia, petechiation, haemorrhages, vascular congestion, oedema and infiltration by inflammatory cells in various tissues (Verwoerd and Erasmus 2004, Darpel *et*

al. 2007, MacLachlan *et al*. 2009, Worwa *et al*. 2010). Affected organs include lymph nodes, spleen, musculature, lungs, heart and the digestive tract. Pulmonary oedema and petechiae or sub-intimal haemorrhages in the tunica of the pulmonary artery near its base are characteristic for fulminant BT but are not pathognomonic (Verwoerd and Erasmus 2004, Darpel *et al*. 2007, MacLachlan *et al*. 2008, MacLachlan *et al*. 2009, Worwa *et al*. 2010). Furthermore, pleural and pericardial effusion, ulcerations and erosions of the mucosa of the upper digestive tract and aspiration pneumonia associated with oesophageal myonecrosis are possible findings of a manifest BTV infection (Verwoerd and Erasmus 2004, Darpel *et al*. 2007, MacLachlan *et al*. 2008, Antoniassi *et al*. 2010).

3.3. Particularities of camelids

Taxonomy and global distribution of South American camelids

Camelidae are even-toed ungulates (Artiodactyla) as are ruminants and pigs, but they belong to the suborder Tylopoda. This family comprises the genera *Camelus* (Old World camelids, OWC), *Lama* and *Vicugna* (South American camelids, SAC) (Fowler 2010a).

Data on the number of SAC worldwide vary considerably in literature and are not documented for every country. Table 1 provides an overview of the estimated distribution of the SAC population.

Table 1. Estimated distribution of the world SAC population (numbers rounded off to the nearest thousand, and number in thousands).

Country or continent	Alpaca	Llama	Guanaco	Vicuña	Total
Australia[3,6,10]	>5–120	<5	-	-	10–125
Europe[1, 2,3,5,10]	34	6–16	<0.1	<0.1	41–51
New Zealand[6]	17	-	-	-	17
North America[1,3,6,9,10,12]	50–215	116–156	<0.245	>0.01	166–371
South America[3,4,6,7,8,10,11]	3,372–5,887	3,564–3,930	584-600	200–276	6,935–9,816
World[3]	3,425	3,680	572	161	7,839
Total	3,461–6,273	3,691–4,107	572–>751	161–>350	7,885–11,482

[1](Kriegl 2004), [2](Kriegl *et al*. 2005), [3](Schwalm 2006), [4](Marín *et al*. 2007), [5](Locher *et al*. 2010), [6](Gunsser 2010), [7](Wolf 2010), [8](Rosadio 2010), [9](Fowler 2010a), [10](Gauly 2011), [11](Haudry De Soucy 2011), [12](Wuliji 2011)

Their global population is estimated about 7.9 to 11.5 million SAC. Most animals are kept in South America (Table 1). Peru is the major alpaca producer in the world keeping about 3.0 to 5.5 million alpacas and 900,000 to 1.2 million llamas (Schwalm 2006, Gunsser 2010, Rosadio 2010, Wolf 2010, Gauly 2011). In Germany, SAC were first established after 1980 (Fowler 2010a, Gauly 2011). Currently, their number is estimated at 5,000 to 7,000 and 15,000 animals according to Locher *et al.* (2010) and Gauly *et al.* (2011), respectively.

Physiological characteristics

Camelids have a unique physiology due to their adaption to hostile environments. SAC became adapted to South American habitats, especially to the high altitude lands of the Andes, while OWC adapted to deal with heat and dehydration in a semi-desert environment (Fowler 2010a).

Camelid erythrocytes are different from RBC of other mammals as they are smaller, very stable, ellipsoid cells without biconcavity (Smith *et al*. 1979, Foster *et al*. 2009, Timm *et al*. 2011). Differences in the erythrocyte membrane proteins and in the organisation of the cytoskeleton have been found, indicating that proteins play an important role in stabilising the camelid elliptocyte (Eitan *et al*. 1976, Omorphos *et al*. 1989). The survival time of SAC erythrocytes is up to 235 days (dependent on the applied method), which is longer than the lifespan of bovine and ovine RBC (Cornelius and Kaneko 1962, Reynafarje *et al*. 1968).

In contrast to ruminants, which have a lymphocytic haemogram (>50% lymphocytes) (Gassmann and Lutz 2010), camelids have a granulocytic haemogram (>50% neutrophil granulocytes) (Wernery *et al*. 1999, Fowler 2010b).

Camelids generally have a reduced susceptibility and a rare clinical outcome of bovine and ovine viral diseases (Wernery *et al*. 1999, Wernery and Kaaden 2004, Kapil *et al*. 2009). They play a minor or negligible role as carriers for important diseases such as foot-and-mouth disease (reviewed by Wernery and Kaaden 2004) or bovine herpesvirus 1 infection (P. König, FLI, unpublished observations). Compared to other mammals, camelids have an exceptional immune system (Wernery *et al*. 1999, Wernery 2001, Conrath *et al*. 2003, Wernery and Kaaden 2004, Vanlandschoot *et al*. 2011) that might play a role in the outcome of diseases. Besides conventional immunoglobulin G (IgG), camelids own heavy-chain antibodies (HCAb; IgG_2 and IgG_3), special subclasses of IgG that lack the light chains and the first constant domain of the heavy chain (C_H1) but have a normal FC region.

The thick layered epitheliochorial placenta of camelids prevents the transplacental transfer of immunoglobulins (Ghazi *et al*. 1994, Wernery 2001, Timm *et al*. 2011). The newborn cria

obtains passive immunity by intestinal absorption of primarily IgG antibodies from the colostrum immediately after birth. Since IgG is predominantly found in camelid colostrum, a selective transfer of IgG similar to that in bovines was assumed (reviewed by Wernery 2001). Stable serum IgG concentrations were found around 4 months after birth, indicating that the immune system has matured (Wernery 2001).

3.3.1. BTV infection in camelids

In camelids, serological evidence for a previous BTV infection was reported on various continents and for different serotypes. Field studies, case reports and animal experiments on BTV infection in camelids are listed in Tables 1 and 2 in Appendix 3. Historically, SAC were considered resistant to BT disease (Rivera *et al*. 1987, Mattson 1994, Afshar *et al*. 1995a) (Table 1 in Appendix 3), while stiffness, dermatitis or trypanosomiasis was occasionally associated with higher seroprevalences in OWC (Table 1 in Appendix 3). The first suspicion of clinical BT disease in SAC was reported by Fowler (1998c). Abortion in a llama concurrently occurred with an episode of respiratory distress, which was followed by a fourfold increase in BTV antibody titre. During the recent BTV-8 and BTV-1 epizootics in Europe, BTV RNA and antibodies were found in a few alpacas and llamas in Germany and France (Henrich *et al*. 2007, Meyer *et al*. 2009, Locher *et al*. 2010), respectively. Furthermore, clinical disease with subsequent fatality or abortion related to BTV infection was reported in a few of the latter SAC and in the USA at about the same time (Ortega *et al*. 2010a). All fatal cases had a brief history of lethargy or weakness, recumbency, anorexia (3 of 4) and acute respiratory distress within 24 hours before death. Necropsy consistently revealed severe congestion and oedema of the lungs (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010a). Additionally, ulcers and erosions in the oral cavity (Henrich *et al*. 2007) and hydrothorax, hydropericardium and acute subendocardial haemorrhages (Ortega *et al*. 2010a) were found in the two alpacas perished in Germany and the USA, respectively. In summary, clinical signs and post-mortem findings were all suggestive of BT (MacLachlan *et al*. 2009, Ortega *et al*. 2010a) (see also II.3.2). BTV RNA was detected in blood and in various tissue samples from the two perished llamas and BTV-1 was isolated (Meyer *et al.* 2009) from lungs and spleens. Interestingly, virus isolation was also isolated from two llama foetuses suggesting transplacental transmission of the BTV-1 field strain, which has never been reported before (EFSA Report 2011).

Despite the high pressure of the two virulent BTV strains in Europe, only these three cases of BTV-infection have been reported in SAC, indicating that BTV-related fatalities occur rather

sporadically in SAC. The negative serological results in a survey of BTV-8 in 354 SAC in Switzerland can be explained by the relatively few BTV cases in ruminants at the time (Zanolari *et al*. 2010b). On the other hand, experimental infection of dromedaries with BTV-1 suggested that Old World camelids can play a potential role in the transmission to *Culicoides*. Hence, OWC could act as reservoirs to carry BTV over long distances and across boarders (Batten *et al*. 2011); similar to wild ruminants (Falconi *et al*. 2011). Whether SAC may play a similar role as OWC remained unknown.

4. Laboratory diagnosis of BTV

Antibody detection

BTV antibodies are generally detectable in blood of domestic and wild ruminants, camels and llamas about 1 to 2 weeks after experimental BTV infection. However, in OWC peak antibody levels were reached relatively late (after 41 dpi) compared to ruminants (10 dpi in sheep, 18 dpi in cattle and 17-28 dpi in red deer) (Afshar *et al*. 1995a, Darpel *et al*. 2007, Dal Pozzo *et al*. 2009a, López-Olvera *et al*. 2010, Batten *et al*. 2011).

Agar gel immunodiffusion (AGID) test have mostly been used for international regulatory purposes (Verwoerd and Erasmus 2004, OIE 2011). However, indirect, double antigen (double recognition, DR) and competitive (c) ELISAs are superior to AGID. They are robust and rapid assays for the detection of group-specific (VP7) antibodies in blood and milk samples with a high specificity and sensitivity (Verwoerd and Erasmus 2004, Kramps *et al*. 2008, Oura *et al*. 2009, Eschbaumer *et al*. 2009, Mertens *et al*. 2009, Mars *et al*. 2010). Furthermore, they have the advantage of species-independent testing of sera. For the detection of vaccinated animals, the cELISA has been considered less sensitive than the DR ELISAs (Oura *et al*. 2009, Eschbaumer *et al*. 2009).

In contrast to the group-specific ELISAs, serum neutralisation tests (SNT) detect serotype-specific (VP2) antibodies against a homologous reference strain. NAbs are thought to be the unique measure to demonstrate protective immunity against re-infection with the homologous BTV serotype *in vitro* after BTV vaccination or natural infection (see also II.5.2.2) (Verwoerd and Erasmus 2004). However, no reports exist about vaccinated animals that were negative in a DR ELISA but were still protected against challenge infection. Furthermore, SNT often yielded false negative results for animals exposed to BTV-8, particularly after vaccination and experimental infection (Worwa *et al*. 2012).

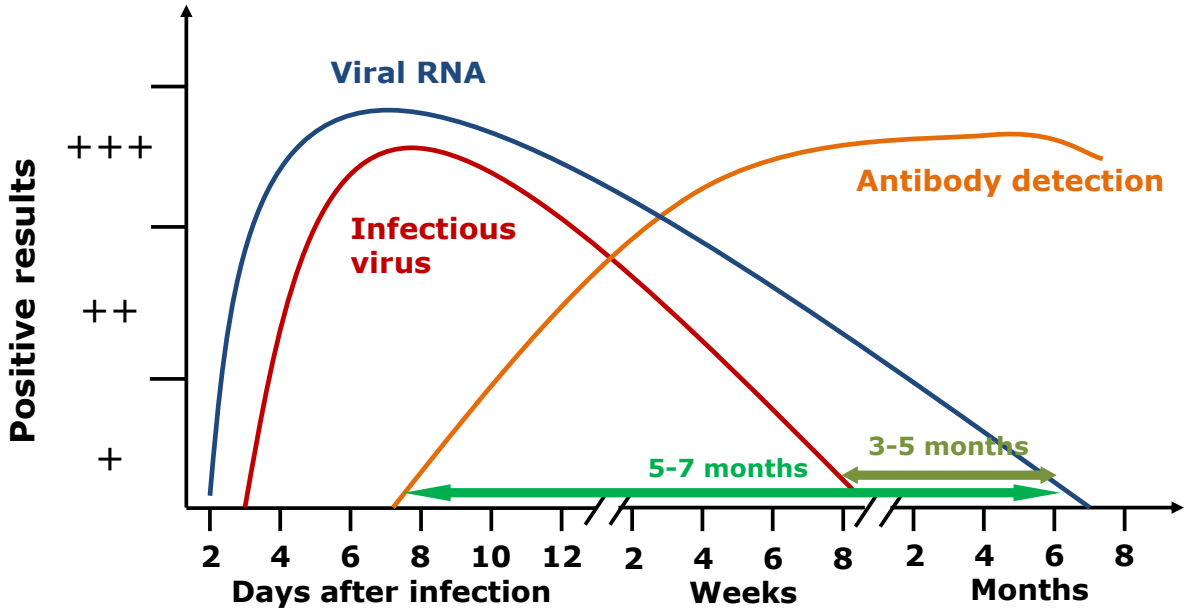


Fig. 9. Timeline of BTV diagnostic parameters. In ruminant blood, BTV RNA and antibodies are detectable at the same time, and viral RNA is detectable long after the end of infectious viraemia. Prolonged viraemia occurs due to the affinity of BTV to blood cells, especially erythrocytes. (Figure source: courtesy of B. Hoffmann and M. Beer, FLI, Greifswald-Insel Riems, and Eschbaumer 2010, modified.)

Virus isolation and genome detection

In ruminant blood, virus isolation and BTV RNA detection are possible as early as 1 to 2 dpi with a subsequent long period of concurrent occurrence with BTV antibodies (see Fig. 9).

Since field strains are usually not well adapted to infection of cell cultures, virus isolation might present difficulties. Consequently, previous passages in embryonated chicken eggs (ECE) may be required for efficient growing in cell culture (Mertens *et al*. 2009, Worwa *et al*. 2010).

At present, virus isolation may be conducted by intravascular inoculation of ECE (Clavijo *et al*. 2000, OIE 2011). Most frequently, a variety of mammalian and insect monolayer cell cultures are used - particularly Vero cells, baby hamster kidney cells (BHK)-21 and KC-cells (Verwoerd and Erasmus 2004, Eschbaumer *et al*. 2010a, Batten *et al*. 2011). IFN alpha/beta receptor deficient ($IFNAR^{-/-}$) mice (Calvo-Pinilla *et al*. 2009a) and *Drosophila melanogaster* (Shaw *et al*. 2012) have recently been established as small animal models for BTV infection in the ruminant and insect host, respectively.

Although virus isolation assays have the inherent advantage to detect live virus and not only the presence of viral genome, they are labour-intensive and requires suitable laboratory

conditions (Mertens *et al*. 2009). Therefore, more sensitive, specific and standardised RT-qPCR or RT-nested PCR assays are now widely used for rapid (automated high-throughput) detection and quantification of very low amounts of BTV RNA (Bonneau *et al*. 2002, Toussaint *et al*. 2007, Hoffmann *et al*. 2009b, Hoffmann *et al*. 2009c). Specific probe designs allow the differentiation of all currently known 26 BTV serotypes (targeting S2) and related topotypes or group specific genome sequences (targeting S5) to distinguish members of other *Orbivirus* species (Toussaint *et al*. 2007, Vandenbussche *et al*. 2009, Hoffmann *et al*. 2009c, Maan *et al*. 2012).

Novel approaches are pushed forward to develop pen-side diagnostic tests enabling on-spot (*in vivo*) diagnosis in the field to improve decision making on preventive measures in case of a disease outbreak (Mertens *et al*. 2009, Saxena *et al*. 2012).

4.1. BTV diagnosis in camelids

Diagnosis of BTV antibodies in camelids and other non-ruminant species is possible using methods that are not species-specific, including cELISA, DR ELISA, neutralisation test (NT) and PCR. Serological (cELISA and NT) and molecular (RT-qPCR and virus isolation on KC-cells) assays have just recently been validated for OWC (Batten *et al*. 2011). However, there is no evidence for a validation of methods to detect BTV infection in SAC, except for Afshar *et al.* (1995a), who validated a cELISA.

5. Prevention and control of BT

BT is a notifiable disease (formerly on List A) of the World Organisation for Animal Health (OIE 2000), according to Council Directive 82/894/EEC (1982) on the notification of animal diseases within the Community, as it can have substantial economic impact (Schwartz-Cornil *et al*. 2008, commission regulation (EC) No 1266/2007). Economic losses are estimated at approximately $3 billion per year (Mellor and Wittmann 2002).

BT monitoring and surveillance programmes have to include serological, virological and entomological surveillance in Member States within and outside of restricted zones (Commission regulation (EC) No 1266/2007, Caporale and Giovannini 2010). However, control strategies differ depending on whether BT disease is endemic in a region or not. While eradication is the usual goal in the latter case, this is not possible in areas where BTV is endemic because of the widely distributed *Culicoides* vectors and the ubiquitous distribution of mammalian reservoir hosts (Verwoerd and Erasmus 2004, Dungu *et al*. 2004).

BTV-8 inheres three particularities compared to other 'wild-type' BTVs (Backx *et al*. 2007, Dal Pozzo *et al*. 2009a, Dal Pozzo *et al*. 2009b) resulting in additional regulations implemented by the European Commission (EC). This serotype may cause serious disease not only in sheep but also in the usually subclinically infected cattle and goats. Frozen stored semen has to be tested free of BTV-8 (Commission regulation (EC) No 1266/2007, Vanbinst *et al*. 2010). The risk of transplacental transmission and the possible spread of the disease by newborn animals in the next vector season have to be precluded. Therefore, all dams have to be immune to BTV before insemination or mating (Commission Regulation (EC) No 384/2008). Furthermore, pregnant animals are effectively restricted in their movement (EFSA Report 2011).

5.1. Vector control

Arbovirus-vector relationships are highly dynamic and extremely difficult to combat (Carpenter *et al*. 2009). The incursion of any BTV serotype in a region is not predictable as *Culicoides* can be transported over long-distances. Furthermore, its establishment in an area depends on the presence of competent vectors, vertebrate hosts and reservoirs. Possible means of protection of hosts from *Culicoides* bites include adulticides such as pour-on insecticides, which are directly administered on livestock (Mellor and Wittmann 2002, Verwoerd and Erasmus 2004, Carpenter *et al*. 2009). However, the application of pyrethroides does not guarantee protection against *Culicoides* bites and subsequent BTV infection (Mehlhorn *et al*. 2008, Schmahl *et al*. 2009). To control *Culicoides* vectors (Verwoerd and Erasmus 2004), modification of habitats, sterilisation of males by irradiation, insecticide-treated netting, residual insecticides and biological agents may be used (Sampson and Gooday 1998, Mellor and Wittmann 2002, Carpenter *et al*. 2009, Calvete *et al*. 2010). Methods for larvae destruction include clearance of potential larval development sites and the use of chemical (American Cyanamid, pyrethroid-based products) or biological (entomopathogenic fungi) larvicides (Mellor and Wittmann 2002, Carpenter *et al*. 2009, Ansari *et al*. 2011).

5.2. BTV vaccination

Vaccination of species susceptible to BTV is considered the tool of choice to control BT and to facilitate safe trade and movement of live ruminants between BTV-affected and BTV-free zones (Giovannini *et al*. 2004b, Savini *et al*. 2008, Gethmann *et al*. 2009, commission regulation (EC) No 1266/2007).

BTV-8 vaccination

Emergency mass vaccination with any available vaccine has been recognised as the most efficient measure for BT control by the European Commission and permits safe trade in live ruminants under certain conditions (Commission regulation (EC) No 1266/2007, Commission Decision 2008/655/EC, Gethmann *et al*. 2009, Caporale and Giovannini 2010). However, considering the numerous drawbacks of modified-live virus (MLV) vaccines discussed in section II.5.2.2, there is disagreement among experts whether MLV vaccines should be used in non-endemic areas at all (Caporale and Giovannini 2010). In non-endemic regions, the common control strategy is eradication (Verwoerd and Erasmus 2004, Dungu *et al*. 2004). Therefore, to preclude natural dissemination of the South African BTV-8 MLV vaccine strain, it was not used during the BTV-8 epizootic in Europe, although this strain has shown a high efficiency against challenge infection with the European BTV-8 (Dungu *et al*. 2008, Caporale and Giovannini 2010).

From spring 2008, the mandatory vaccination programs were successfully implemented using five monovalent inactivated BTV-8 vaccines: Bluevac®-8, CZ Veterinaria, Spain; BTVPUR® AlSap 8, Merial, Lyon, France; Zulvac® 8 Bovis and Ovis, Fort Dodge, The Netherlands and Bovilis® BTV 8, Intervet, Boxmeer, The Netherlands (FLI 2009, Zientara *et al*. 2010, Gethmann *et al*. 2010, EU-BTNET system 2010b, Oura *et al*. 2012). In Germany, all but the Bovilis vaccine were used. An overview of vaccination campaigns against BTV-8 in Europe from 2008 to 2011 is given in Table 1 in Appendix 2.

Vaccination coverage of over 80% of the susceptible ruminant population significantly reduces BTV circulation, and allows safe movement of ruminants from BTV-affected to BTV-free areas (Caporale *et al*. 2004, Patta *et al*. 2004, Giovannini *et al*. 2004a, Giovannini *et al*. 2004b, 2004c). In Germany, a vaccination coverage of over 80% had been achieved by the end of 2008 (Gethmann *et al*. 2010).

Most countries in Europe have reverted to voluntary vaccination campaigns in 2011 (Table 1 in Appendix 2) (Oura 2011), or vaccination was prohibited in areas declared free of BTV (*Anon*. 2010b). Since that time, no major BTV outbreaks have been recorded in Europe, although vaccine coverage has dramatically decreased (Oura 2011). Hence, the risk of a re-introduction of BTV-8 has been assessed as equally high than the risk of introduction of other BTV serotypes (FLI 2012). Vaccination before a BTV incursion occurs into a naïve population has various advantages. Therefore, the EU recently allowed the use of inactivated vaccines within a BT free area, given that the Member States authorises its use (DEFRA 2012).

5.2.1. Types of BTV vaccines

In general, three types of vaccines against BTV can be distinguished: live attenuated vaccines, live recombinant virus-vector vaccines and non-replicating vaccine/adjuvant combinations. The latter are either inactivated whole virus preparations, virus-like particles (VLP), core-like particles (CLP) or subunit vaccines (Roy *et al*. 1990, Lobato *et al*. 1997, Boone *et al*. 2007, Savini *et al*. 2008, Alpar *et al*. 2009, Calvo-Pinilla *et al*. 2009b, Franceschi *et al*. 2011). The five BTV-8 vaccines that have been used in Europe are inactivated, monovalent BTV-8 preparations in aqueous solution. The seed viruses for these vaccines were European BTV-8 isolates from the 2006 outbreak in Northern Europe, which were replicated in BHK cell cultures. To enhance the immune response, aluminium hydroxide and saponin were used as adjuvants (Gethmann *et al*. 2009, Eschbaumer *et al*. 2009, Bruckner *et al*. 2009b). Vaccination programs for the control of BT in ruminants have limitations due to difficulties in differentiating infected from vaccinated animals. To overcome this problem, recent developments target marker vaccines and test systems that differentiate infected from vaccinated animals (DIVA). This is possible as vaccinated animals do not elicit a detectable antibody response to NS of BTV (Alpar *et al*. 2009, Barros *et al*. 2009).

5.2.2. Safety and efficacy of live-attenuated and inactivated BTV vaccines

Currently, only MLV and some inactivated vaccines are available under European Community approved national disease control programs (Savini *et al*. 2008). MLV as well as inactivated BTV vaccines have a high level of efficacy to challenge infection, preventing clinical disease and viraemia in sheep, goats, cattle and red deer (Di Emidio B. *et al*. 2004, Savini *et al*. 2004b, 2008, Eschbaumer *et al*. 2009, Savini *et al*. 2009, Wäckerlin *et al*. 2010, Bréard *et al*. 2011, Lorca-Oró *et al*. 2012). In MLV, however, the level of safety varies considerably according to the used formulation, BTV serotypes and number of serotypes included in the vaccine (Savini *et al*. 2008). Adverse effects include clinical BT disease (Veronesi *et al*. 2010) and depressed milk production (Savini *et al*. 2004c). Abortion/embryonic death and teratogenesis can occur when these vaccines are used in pregnant females (Flanagan and Johnson 1995, Savini *et al*. 2008). Viraemia is a common finding after vaccination with MLV (Savini *et al*. 2008), and natural dissemination of vaccine strains of BTV-16 (Monaco *et al*. 2006) and BTV-2 (Ferrari *et al*. 2005) has already been documented in Europe. Furthermore, MLV have the potential of reassortment (Batten *et al*. 2008, Maan *et al*. 2010) and an increase of or reversion to virulence (Batten *et al*. 2008, Alpar *et al*. 2009, Veronesi *et al*. 2010).

Therefore, inactivated vaccines have been chosen for the massive compulsory vaccination programs against BTV-8 in Europe – despite high production costs and the need for booster vaccination to elicit and maintain a protective immunity, especially in cattle (EFSA Report 2007, Savini *et al*. 2008, Gethmann *et al*. 2009, Zientara *et al*. 2010).

No or only minor adverse effects and a high level of efficacy (antibody response, prevention of clinical signs and viraemia) to challenge infection with the homologous BTV serotype were observed in domestic ruminants up to three years after vaccination with inactivated BTV vaccines against BTV-2, -4, -8, and -16 (Di Emidio B. *et al*. 2004, Savini *et al*. 2007, Savini *et al*. 2008, Eschbaumer *et al*. 2009, Gethmann *et al*. 2009, Savini *et al*. 2009, Oura *et al*. 2009, Bruckner *et al*. 2009a, Hamers *et al*. 2009a, Hamers *et al*. 2009b, Wäckerlin *et al*. 2010, Tschuor *et al*. 2010, Bréard *et al*. 2011, Oura *et al*. 2012). Adverse effects mainly included a transient rise in body temperature and local swelling at the injection site (Gethmann *et al*. 2009, Bruckner *et al*. 2009a, Bréard *et al*. 2011).

Bivalent inactivated vaccines against BTV serotypes 1 and 8 have only recently been introduced to the market (Veronesi *et al*. 2010). Good tolerance was also found for the Zulvac®1+8 Bovis vaccine in a safety study in 3-month-old calves (EMA 2010a, 2010b). The granting of marketing authorisation has been recommended by the European Medicines Agency, and the vaccine is currently pending the decisions of the European Commission (EMA 2012). Currently, inactivated vaccines against the BTV serotypes 1, 2, 4 and 8 have marketing authorisation at the EU level (DEFRA 2012).

NAbs play a key role in protecting animals from disease and viraemia following infection with the homologous wild-type BTV. Therefore, vaccines target the NAb response as a major tool of protective immunity (Alpar *et al*. 2009). However, protective immunity has also been observed in vaccinated animals without detectable NAbs (Savini *et al*. 2004b, Eschbaumer *et al*. 2009, Savini *et al*. 2009). Therefore, the degree of protection depends on several interacting factors and possibly includes cell-mediated (T-cell determinants and CTL) immune response (Jeggo *et al*. 1984, Andrew *et al*. 1995, Alpar *et al*. 2009, Savini *et al*. 2009, Hund *et al*. 2012).

5.2.3. BTV vaccination of camelids

Although SAC are susceptible to BTV (Rivera *et al*. 1987, Mattson 1994, Afshar *et al*. 1995b, Fowler 1998b, Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b), they were generally not included in the mandatory vaccination programs in Europe. Data about BT in SAC were sparsely documented (Mattson 1994). However, fatalities in SAC and the detection

of BTV upon post-mortem examination were recently reported (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b). Therefore, veterinary authorities were confronted with the question whether SAC should better be vaccinated after all. To reduce the number of naïve susceptible animals and to minimize the risk of BTV spread and virus circulation, vaccination of SAC should be considered in vaccination campaigns. However, manufacturers did not give recommendations for SAC, and no information about dosage, immunogenicity and safety of any BTV vaccine was available. In a recent study, good safety and immunogenicity have been documented in SAC. Similar to ruminants, SAC (n=42) vaccinated with any one of the two inactivated BTV-8 vaccines BTVPUR® AlSap 8 or Bovilis® BTV8 only presented moderate local swellings at the injection site and a slight increased body temperature in all animals after the first and the booster vaccination (after 21 days) (Gethmann *et al*. 2009, Zanolari *et al*. 2010a). BTV-vaccination of two dromedaries resulted in seroconversion after subcutaneous (s.c.) vaccination with twice 4 ml of BTVPUR® AlSap 2-4 OWC (2008). However, the long-term progression of BTV antibodies until booster vaccination one year after initial immunisation has so far not been evaluated in SAC.

Registered vaccines cannot be used off-label in Germany. During the time of the study the BTV-8 vaccines had not yet obtained marketing authorisation (Directive 2001/82/EC, Saegerman *et al*. 2007, BMELV 2010) and could therefore be used for SAC. With BTV vaccines now authorised (see II.5.2.2), the use of those vaccines in SAC is no longer possible.

For a successful vaccination regimen it is fundamental to know how long colostral immunity persists (Savini *et al*. 2004a, Vitour *et al*. 2011). Interference of colostrum-derived neutralising BTV-8 antibodies with antibody production after challenge infection and vaccination was reported in lambs and calves, respectively, from BTV-8 vaccinated dams (Oura *et al*. 2010, Vitour *et al*. 2011). However, passive immunity against BTV has not been monitored in crias.

III. OBJECTIVES

BTV-8, an arbovirus causing severe haemorrhagic disease in ruminants, was the first-ever BTV serotype that emerged to northern Europe. Approximately 89,000 BTV-8 outbreaks have been registered in Europe from 2006 until the end of 2010. Since its incursion, many studies have been carried out to investigate the epidemiology and pathogenesis of BTV-8 infection in ruminants as well as the *Culicoides* fauna including vectors of BTV. Safety and immunogenicity of inactivated BTV-8 vaccines have been examined, and diagnostic methods have been developed and optimised for BTV-diagnosis in ruminants. However, no studies were conducted to investigate the pathogenesis and prophylaxis of BTV infection in SAC although they are susceptible to BTV.

For about a decade, SAC have become increasingly popular in Germany. They are frequently kept next to ruminants on the same farm. Hence, there was a particular risk for transmission of this disease between ruminant and camelid species. However, the role of SAC in the epidemiology of BTV - as possible reservoir or risk factor for ruminant livestock - was unknown.

The objectives of the presented work were to examine the epidemiology, pathogenesis and immunogenicity of BTV-8 infection in SAC and to investigate the immune response elicited in SAC after BTV-8 vaccination. Therefore, the following studies were conducted:

(i) Entomological monitoring on three SAC farms in Germany (2008-2009) with emphasis on the abundance, seasonal dynamic and breeding habitats of *Culicoides* spp. incriminated as BTV vectors. Analysis of selected midge samples for BTV genome.

(ii) Large-scale serological and virological cross-sectional study of BTV infection in SAC herds all over Germany to document prevalence, morbidity and lethality rates in SAC at the end of the BTV-8 epizootic in Germany (2008/2009).

(iii) Experimental BTV-8 infection of llamas and alpacas and a blood-cell binding experiment to obtain new insights in the pathogenesis of BTV-8 infection and the role of SAC in the epidemiology of BTV. Validation of serological and virological methods for BTV diagnosis in SAC with samples obtained from the animal experiment.

(iv) Vaccination study under field conditions to evaluate vaccine tolerance, immune response and long-term antibody development in SAC vaccinated with inactivated BTV-8 vaccines. Monitoring of colostral BTV-antibodies in crias from BTV-8 vaccinated dams.

IV. MATERIAL AND METHODS

1. Entomological monitoring

1.1. Trap locations

The population dynamic of ceratopogonids was monitored on three SAC farms in Germany. The farms were located in areas where BTV prevalence in ruminants was either high (southern Hesse and southern Lower Saxony in central Germany) or low (southern Bavaria, region Allgäu, in southeastern Germany) in the years 2006 to 2008 (Conraths *et al*. 2009). The selection of the study farms was conducted before knowing the BTV infection status of the herds. In the cross-sectional study, several SAC in these herds were found positive for BTV antibodies but negative for BTV RNA in autumn 2008 (Fig. 10). The highest BTV seroprevalence was found on the Hessian farm (farm A) (Table 2). See Fig. 10 for trap locations in Germany and Table 2 for information on the surrounding landscape and animal husbandry on the three farms. Farm names are labelled according to Table 2.

1.2. Trapping protocol

Biting midges were caught in black-light traps with a suction fan (Fig. 11; BG-Sentinel^{TM} UV light, Biogent, Regensburg, Germany) from July 2008 to June (all farms) or November 2009 (farms B and C). On each farm, one trap was set up on pasture ("outdoor") and in the stable ("indoor") each, at a height of about 1.5 to 2 meters.

All traps were activated in the first 7 consecutive nights of each month by a photosensitivity switch from dusk until dawn at a light intensity below 20 lx (Clausen *et al*. 2009, Hoffmann *et al*. 2009a). In winter, outdoor traps were not activated from December 2008 to March 2009.

Biting midges were caught in catch beakers with 70% ethanol and were harvested once a day during the respective catching period. Midges were preserved in 70% ethanol until further analyses.

On farm A, an emergence trap (Fig. 12; Bodeneklektor: 100 x 100 cm, Fiebig Lehrmittel, Berlin, Germany) was set up on fresh to several-day-old llama faeces ('faeces trap') from April to September 2009 Llamas generally defecate and urinate on the same dung pile (Fowler 1998d). The selected dung pile has been used and regularly cleaned for around 10 years. For comparison, a second emergence trap was set up on a dung-free area on the same pasture ('pasture trap') from May to September 2009. Hatched biting midges attempting to

escape through the tent roof of the emergence trap were caught in a catch beaker with 70% ethanol. Similar to the black-light traps, the emergence traps were also operated in the first week of each month, but catch beakers were not replaced during these periods.

Table 2. Locations of BG-Sentinel™ UV light traps in Germany.

Location (farm label)	Hesse (A)	Lower Saxony (B)	Allgäu in Bavaria (C)
Altitude a.s.l.[a]	300 m	185 m	807 m
Landscape	Small valley, mixed forest	Plane landscape of hills	Plane landscape of hills, prealpine lands
Land use	Pasture, forestry; > 2 km: cultivated landscape, viticulture	Pasture, cultivated landscape	Pasture, cultivated landscape
Waters or marshland (distance)	stream alongside premises	Trout ponds (300 m)	Marshland (200 m)
Number of SAC (BTV-seropositive[b])	100 (38%, 21 of 55)	20 (5%, 1 of 20)	122 (2%, 1 of 54)
Number of ruminants in vicinity (approximate distance)	15 goats (600 m)	220 sheep (300 m), 300 cattle (300 m)	10 sheep (150 m), 330 cattle (150 to 500 m)

[a] a.s.l., above sea level, [b] in autumn 2008

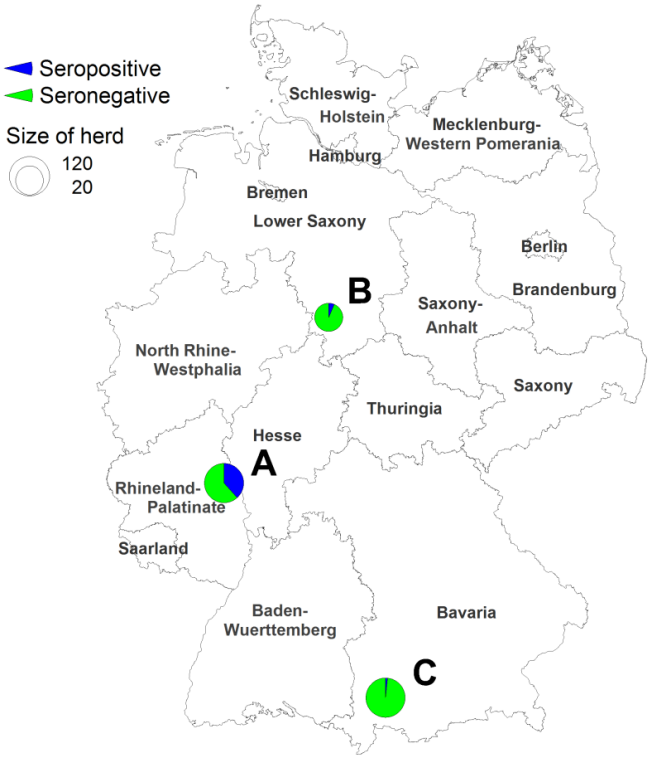


Fig. 10. Map showing locations of the black-light traps set up on three South American camelid farms in Germany 2008/2009. Dark blue wedges show proportions of seropositive SAC.

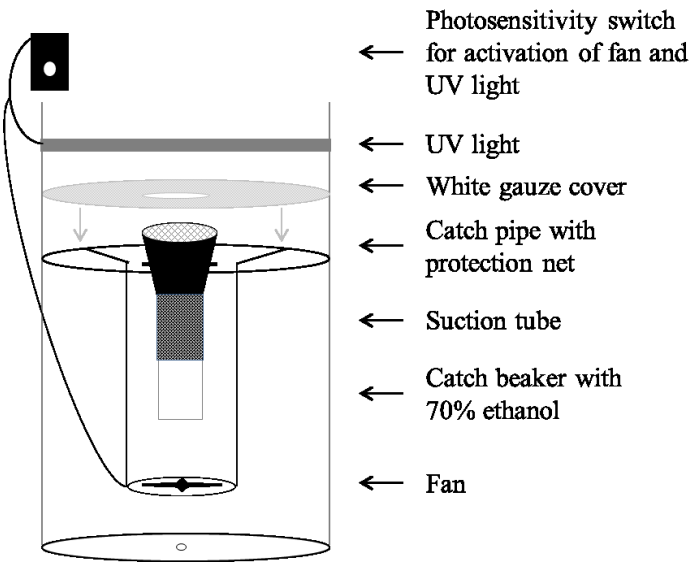


Fig. 11. BG-Sentinel™ UV light midge trap. Insects are attracted by black light and sucked into a catch beaker by fan (Mehlhorn *et al*. 2009a).

Fig. 12. Emergence trap. Emerging insects are caught in a catch beaker on top of the trap.

1.3. Morphological identification of ceratopogonids

All ceratopogonids were sorted from catches, except for larger samples. In this case, a maximum of 500 individuals were sorted according to Van Ark and Meiswinkel (1992) to obtain a representative sample of collected species. Ceratopogonids were morphologically identified to genus and those of the genus *Culicoides* to complex level or, if possible, to species level (Campbell and Pelham-Clinton 1960, Delécolle 1985, Glukhova 1989, 2005, IAH 2009). Females of the Obsoletus complex (in Germany: *Culicoides obsoletus s.s.*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) cannot be reliably identified to species based on morphological characters, but males may be differentiated by their genitalia (Vilar *et al*. 2011). Therefore, males of this complex were embedded in Canada balsam for microscopical inspection of their genitalia and species identification. In specimens where no males were caught by emergence trap, females belonging to the cryptic species of the Obsoletus complex were processed by species-specific diagnostic PCR assays (Mathieu *et al*. 2007, Lehmann *et al*. 2012).

1.4. Weather data

Electronic time-series measurements of temperature (°C) and relative humidity were conducted on pasture and in the stable with the HOBO H8 Pro Series logger™ (Onset

Computer Corporation, Bourne, USA) every 4 hours from May to June (all farms) or November 2009 (farms B and C). The weather stations were positioned close to the traps at the same heights.

2. Cross-sectional study

2.1. Study design and sampling

The study population was selected by multi-stage sampling. Among a total of 227 contacted farms in Germany, most (n=170) had been systematically selected by postal code from a mailing list of an association of breeders, owners and friends of SAC[3] (NWK e.V.) from summer 2008 to spring 2009. The other 57 owners were contacted at meetings of other SAC associations and after recommendation by other participants. All owners with at least eight SAC that agreed to participate in the cross-sectional study (n=91) were visited once, and individual SAC were randomly selected. Three farms that kept less than eight SAC were also accepted in the study as they were located in regions where only a few herds were available.
For sample size calculations a BTV prevalence of 5% was assumed (Conraths *et al*. 2009). When the study was designed, no exact data on the number of SAC in Germany were available. Based on an estimate of 5,000 SAC in Germany in 2004 (Rohbeck 2006), a current population size of 10,000 animals was assumed. The overall sample size necessary to detect a 5% BTV (sero-)prevalence (Conraths *et al*. 2009) with 95% probability and an accepted error of 1% was calculated (Cannon and Roe 1982) to be at least 1,544. The number of blood samples to take per farm was calculated from the size of the SAC herd. A maximum sample size of 59 for large herds was required to detect at least one serologically or virologically BTV-positive animal in a herd with a 95% probability (Cannon and Roe 1982). See Table 3 for the number of SAC tested per herd. Blood samples were taken from 1,742 (67% of 2601) unvaccinated SAC (1,249 alpacas, 479 llamas, 14 SAC of other breeds) on 91 farms located in all federal states of Germany, except city states (Fig. 18, Table 8; and Table 1 Appendix 4). In general, non-weaned crias from sampled dams also participated in the study. All samples were taken between mid-September 2008 and early May 2009. Most herds (91%) were visited from November 2008 to March 2009, outside the main BTV vector season.

[3]Verein der Züchter, Halter und Freunde von Neuweltkameliden e.V., http://www.lamas-alpakas.de

Table 3. Number of SAC per herd tested for BTV-8 antibodies in Germany 2008/2009, number of farms on which sample sizes were lower than required assuming a 5% prevalence with 95% probability (Cannon and Roe, 1982) and median no. of missing samples per herd.

No. of tested SAC per herd	No. of examined herds	Proportion of herds (%)	No. of herds with lower sample size	Median no. of missing samples per herd
<8	7	7.7	1	1
8-10	18	19.8	4	1
11-20	41	45.1	25	2
21-40	14	15.4	6	13
41-60	11	12.1	0	-
Total	91	100	36	2

2.2. Questionnaire

Information about the size of the herd and the presence of ruminants on farms was collected by telephone interview (Appendix 5). Of particular interest were observations of BT disease or fatalities in any SAC on their farms. A separate questionnaire (Appendix 6) was sent to the owners before the farm visit to collect data on species, breeds, sex, age, pedigree (relation between crias and dams) and housing type (pasture or paddock with access to a shelter during the day and/or night, pasture or paddock only, stable only) for SAC. Date and origin of purchased animals were recorded to preclude BTV infection at a different location than the visited farm. Missing or doubtful data were completed during the farm visit. The telephone interviews and mailed questionnaires were handled by one researcher.

3. Animal experiment

3.1. Animals and experimental design

Three alpaca males (*Vicugna pacos*; age: 8, 15 and 18 months) and three llama males (*Lama glama* age: 7 months) were obtained from German breeders in January 2010. To exclude a previous exposure to BTV, serum and whole blood samples were examined with DR ELISA and RT-qPCR (see sections IV.5 and IV.6). Blood collection and animal transport were conducted outside the main vector season, when a BTV infection is very unlikely to occur (Wilson *et al*. 2007). Seven days before the experiment, all animals were housed in species groups in the containment facility of the Friedrich-Loeffler-Institut (FLI), Isle of Riems, Germany, with a 12 h light regimen. Feed and water were provided *ad libitum*. Blood samples

were taken one day before the experiment to evaluate haematological parameters and to prove them free of BTV antibodies and BTV RNA (see respective sections below).

For BTV infection, the SAC were subcutaneously injected at multiple sites in the freshly clipped shoulder and dorsal thorax regions. Each animal received 10^5 TCID50 (50% tissue culture infective doses) of a recent German BTV-8 isolate (Eschbaumer *et al*. 2009) in 4 ml of tissue culture supernatant diluted in phosphate-buffered saline (PBS). Briefly, a Holstein calf had been inoculated with pooled blood from a BT outbreak in sheep. Virus was isolated from the calf and passaged twice on Vero cells (RIE15, Collection of Cell Lines in Veterinary Medicine, FLI Insel Riems).

The inoculum used for the SAC contained 5 x 10^8 BTV-8 genome copies per ml (quantification cycle [C_q] value 18.8). It was proven to be free of any bacterial contamination.

After inoculation, whole blood and serum samples were taken at regular intervals. EDTA blood was stored at 4 °C, and serum samples were harvested within 4 h of collection, inactivated at 56 °C for 30 min, and stored at 4 °C until analysis.

Only three animals per species were available. Because three replicates were considered the absolute minimum for a meaningful experiment, it was decided to inoculate all animals and not to use a control group.

3.2. Clinical and haematological parameters

During the entire study, rectal body temperatures and close clinical monitoring were performed daily from one day before to 15 dpi (Fowler 1998a, Hengrave Burri *et al*. 2005). This included examination of the eyes, conjunctival membranes, oral and nasal mucosa, auscultation of the heart and lungs, palpation of mandibular, axillar, cervical, inguinal and popliteal lymph nodes, examination of the skin of the coronary band and interdigital spaces for lesions and increased temperature. Blood samples taken on days -1, 2, 6, 8, 10, 13, 16, 20, 24, 28 and 35 were tested in a Cell-dyn® 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL, USA) using the 'goat' analysis profile.

3.3. Post-mortem examination

Euthanasia and post-mortem examination of all SAC was performed on 106 dpi. All animals were sedated by intramuscular (i.m.) application of 0.5 ml (llamas) or 0.3 ml (alpacas) of 1% detomidine (Cepesedan® RP, CP-Pharma, Burgdorf, Germany) and led to the necropsy room. For anaesthesia, each animal received an i.m. injection of 250 mg xylazine powder (Rompun® TS, Bayer HealthCare, Leverkusen, Germany) dissolved in 2 ml of 10% ketamine

(Medistar, Ascheberg, Germany) ("Hellabrunner Mischung"; Wiesner 1998). Then they were euthanized by intravenous injection of 25 ml of T61® (Intervet, Unterschleißheim, Germany) and exsanguinated through the jugular veins.
Spleen material of all SAC was collected for RT-qPCR analysis and for virus isolation in IFNAR$^{-/-}$ mice and tissue culture (see respective sections).

3.4. Blood-cell binding experiment

For the blood-cell binding experiment, EDTA blood samples were obtained from eight cattle (kept in the isolation facilities of the FLI, Isle of Riems), three pigs and six SAC (three alpacas and three llamas) (kept in the containment facility of the FLI, Isle of Riems), all naïve to BTV. As there are no reports of BTV infection in pigs, they can be considered not susceptible to BTV. Therefore, blood samples of pigs served as unrelated controls. Two tests were set up in parallel: In the first experiment, virus was added to 1 ml unmodified blood, in the second experiment 1 ml blood was washed twice and reconstituted to its original volume with PBS. In both cases, the blood was then spiked with 10^4 $TCID_{50}$ of BTV-8. All tubes were mixed thoroughly and incubated at 37 °C for 2 h on a rocking table. Specimens were taken from the whole sample first, then separately from supernatant and blood cells after centrifugation (1 min at 1,500 g). The remaining blood cells were washed 5 times with PBS. Another sample was taken from the washed suspension; again cells and supernatant were separated by centrifugation and sampled independently. All samples were diluted in 75 µl of MEM supplemented with 10% fetal bovine serum (free of BTV antibodies). The BTV RNA load of all samples was then quantified as described earlier.

4. Bluetongue vaccination

4.1. Vaccination study: Animals and study design

In this vaccination study, 48 alpacas (*Vicugna pacos*) and 17 llamas (*Lama glama*) from 5 herds were vaccinated with one of three inactivated BTV-8 vaccines (BLUEVAC®-8, CZ Veterinaria, Spain; BTVPUR® AlSap 8, Merial, Lyon, France; Zulvac® 8 Bovis, Fort Dodge, The Netherlands) used in the 2008 and 2009 BT vaccination campaign in Germany (Gethmann *et al*. 2009). The vaccines are labelled according to Table 4. Of the 65 vaccinated animals, one quarter (15 alpacas and 2 llamas) were crias between 2 and 8 months of age (Table 4). In all herds, blood samples were taken before vaccination to prove the animals free of BTV antibodies. Vaccines were applied s.c. according to the manufacturers' instructions

(see Table 4). Fort Dodge recommends i.m. application of their vaccine, but herd 5 was also vaccinated s.c. Vaccination was conducted between mid-September and December 2008. The first post vaccination (p.v.) samples were collected from mid-November 2008 to the end of January 2009, outside the main BTV vector season (Baldet *et al*. 2008, Hoffmann *et al*. 2009a). All farms were located in regions of Germany where BTV-8 cases had never been registered (Conraths *et al*. 2009, EU-BTNET system 2009).

In herd 3, six unvaccinated alpacas served as negative controls. Crias in herd 2 and 5 that became seronegative after the elimination of detectable colostrum-derived antibodies served as sentinels for vectorborne introduction of BTV into the herds.

Our influence on the study design was limited because only 5 of 227 owners contacted by telephone agreed to participate. The vaccinating veterinarian was chosen by the owner, the used vaccine was selected by the local veterinary authorities or by the veterinarian, and the dosage was decided by the veterinarian. Negative controls or sentinel crias were only available in two herds. Nevertheless, natural BTV infections after vaccination are very unlikely based on the location of the farm in a BTV low risk area.

Table 4. Animals, vaccines and dosages used in the BTV-8 vaccination study.

Herd #	Species	No. of SAC	Vaccine[a]	Vaccine label	Dosage (s.c.)	Relative dosage	Second dose	Age (y)	Sex (m/f)
1	Alpaca	14	BLUEVAC®-8	C	2 x 2 ml	2 x sheep dose	After 4w	3 - 12	5/9
2	Alpaca	15	BLUEVAC®-8	C	2 x 2 ml	2 x sheep dose	After 3w	0.2 - 5	6/9
3	Alpaca	19	BLUEVAC®-8	C	1 x 2 ml	Sheep dose	None	0.3 - 12	11/8
4	Llama	9	BTVPUR® AlSap 8	M	2 x 1 ml	Cattle dose	After 4w	1.5 - 15	3/6
5	Llama	8	Zulvac® 8 Bovis	F	2 x 2 ml	Cattle dose	After 3w	0.6 - 7	3/5

[a] Lot numbers available upon request; s.c., subcutaneous; m, male; f, female; p.v., post vaccination (after the completed vaccination); w, weeks; y, years; C, CZ Veterinaria; M, Merial; F, Fort Dodge.

4.2. Tolerance of BTV-8 vaccines

Age, sex and species were recorded for the 65 vaccinated (Table 4) and 6 unvaccinated SAC that participated in this study. Furthermore, data on vaccination regimens and vaccines used for another 210 alpacas and 72 llamas on 26 farms were collected by telephone questionnaire. The owners of all 347 vaccinated SAC were asked retrospectively to report any adverse effects after BTV-8 vaccination including fatalities, swelling at the injection site, other

observed abnormalities concerning the general condition (all farms) or increased abortion rates (farms 1 to 5).

4.3. Colostral transfer of maternal BTV antibodies

To study the transfer and progression of maternal BTV antibodies in crias from BTV-8 vaccinated dams, sera from eight crias of herd 2 and two crias of herd 5 were repeatedly examined by ELISA from 1 to 19 weeks after birth (post natum, p.n.) until BTV antibodies were no longer detectable (Fig. 25). All dams were vaccinated according to Table 4, except the mother of cria 10. This dam was vaccinated with two 1 ml doses of vaccine M, and a sample before the vaccination was not available. Seven crias of herd 2 (alpaca crias 1 to 7) were born in 2009, 5 to 9 months after the second vaccination of their mothers. In 2010, 12 months after the birth of cria 7, the same dam gave birth to an additional cria (8), 18 months after the completed vaccination. The two llama crias (9 and 10) of herd 5 were born in 2009, 4 and 8 months after the second vaccination of their mothers.

5. Serology

Blood was drawn by jugular puncture and collected in plain tubes and tubes with potassium EDTA. Serum was harvested and stored at -20 °C, while EDTA-treated blood was stored at 4 °C until further analysis.

5.1. Enzyme-linked immunosorbent assay

All sera were analysed in commercially available serogroup-specific, but not species-specific ELISAs that determine antibody levels against the BTV major core protein VP7. The tests were carried out according to the manufacturers' instructions. Positive and negative controls provided with the test kits were used to determine the cut-off values, and reference sera from cattle confirmed the successful execution of each tests. The double recognition (DR) ELISA (INGEZIM BTV DR 12.BTV.K0, INGENASA, Madrid, Spain or PrioCHECK® BTV DR, Prionics Deutschland GmbH, Planegg-Martinsried, Germany, respectively[4]) and the ID Screen® Blue Tongue Competition ELISA (cELISA) (ID VET, Montpellier, France) were validated with samples obtained from the animal experiment (see below).

After a preliminary comparative analysis of five different ELISA systems (data not shown) (see Wäckerlin *et al*. 2009), 1742 sera of unvaccinated SAC were analysed in the DR ELISA.

[4] The INGEZIM BTV DR test was developed by Ingenasa and is distributed in Germany by Prionics GmbH under their own name. The tests are identical.

Serological data of the animal experiment were collected from samples taken on -1, 2, 6, 8, 10, 13, 16, 20, 24, 28, 35, 48, 62, 70, 77, 83, 90, 97 and 106 dpi using the DR ELISA and the cELISA.

From all owners that participated in the vaccination study prior permission was obtained to collect blood samples. Antibody levels from the vaccinated animals were measured from 3 to 4 weeks up to 1 or 1.5 years after the completed immunisation (single or twofold vaccination, Table 4). All serum samples (including pre-immunisation sera of all herds) were tested with the DR ELISA. In addition, the pre-immunisation sera of three herds (1, 2 and 4) and the 1st sample after the completed vaccination of two herds (1, 4) were tested in a competitive ELISA (VMRD ELISA, Bluetongue Virus Antibody Test Kit, VMRD, Pullman, WA, USA). Nine dams and 10 crias from herds 2 and 5 were also tested by SNT that was validated with samples obtained from the animal experiment (Schulz *et al*. 2012b) (see IV.5.2).

5.2. Serum neutralisation test

SNTs against the BTV-8 isolate used in the experiment were performed according to the standard protocol of the Community Reference Laboratory for BT at the Institute for Animal Health, Pirbright, UK. Briefly, inactivated serum samples were diluted tenfold in Dulbecco's Modified Eagle Medium with 25 mM HEPES, 4.5 g/l glucose and antibiotics (DMEM). From this initial dilution, sera were serially diluted twofold to 1/1280 in DMEM, and 100 µl of each dilution were added to 96-well plates in duplicate. A total of 100 $TCID_{50}$ of BTV-8 in 100 µl of DMEM were added to each well. After incubation for one hour at 37 °C, plates were stored at 4 °C overnight. The next day, 10^4 Vero cells in 50 µl of RPMI-1640 medium with 5% foetal bovine serum (free of BTV antibodies) and antibiotics were added to each well. Plates were incubated for 7 days at 37 °C and scored for cytopathic effect at 5 and 7 dpi. The neutralising doses given are the reciprocal of the serum dilution that caused virus neutralisation in 50% of the replicates (ND_{50}), and were calculated according to the method of Spearman and Kärber (Mayr *et al*. 1974).

The first sample from each cria and samples taken from their mothers 3 weeks p.v. and around parturition (Table 13) were also tested by this SNT beginning with a twofold dilution of the sample.

6. Virological analyses

6.1. Extraction of viral RNA

Selected *Culicoides* midges caught in black-light traps in the night of July 1/2, 2008 and September to November 2008 indoor and outdoor on farm A as well as in the night of July 1/2, 2008 indoor on farms B and C were sorted by Obsoletus and Pulicaris complexes as well as "other" *Culicoides* spp.

For RNA extraction, batches of ≤50 female *Culicoides* midges were prepared (Hoffmann *et al*. 2009a). Each batch was homogenised in 400 µl lysis buffer (NucleoSpin® 96 RNA kit, Macherey-Nagel, Düren, Germany) with three steel beads (3 mm) using TissueLyser (Qiagen, Hilden, Germany) for 2 min at 30 Hz and centrifuged for 1 min at maximum speed. Nucleic acids were extracted with an extraction robot (MagNA Pure® LC, Roche Diagnostics, Mannheim, Germany) with the MagNA Pure® LC Total Nucleic Acid Isolation Kit (Roche) and eluted in 100 µl.

All EDTA-treated blood samples of SAC that were seropositive in the prevalence survey by DR ELISA were analysed for BTV RNA. Total RNA was extracted manually using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) or by an extraction robot (MICROLAB® STAR, Hamilton, Bonaduz, Schweiz) with the NucleoSpin® 96 RNA Kit (Macherey-Nagel, Düren, Germany). RNA was eluted in 50 µl (Qiagen kit) respectively 100 µl (Macherey-Nagel kit).

For samples obtained from the animal experiment, viral RNA was extracted from whole blood, serum (2 to 62 dpi) or washed blood cells (2 to 13 dpi). Washed blood cells were obtained from 1 ml whole blood and were resuspended in PBS. RNA was extracted from 75 µl of whole blood, 140 µl of serum and 100 µl of blood cell suspension with the QIAamp® Viral RNA Mini Kit and was eluted with 50 µl. At the end of the experiment, samples from 2 to 62 dpi were extracted again with an extraction robot (2 to 35 dpi: MICROLAB® STAR, Hamilton, Bonaduz, Schweiz; and 48 to 62 dpi: MagNA Pure® LC, Roche Diagnostics, Mannheim, Germany) with the NucleoSpin® 96 RNA Kit and the MagNA Pure® LC Total Nucleic Acid Isolation Kit (Roche), respectively. With these kits RNA was extracted from 100 µl of each sample and eluted in 100 µl.

For manual extraction of RNA from the SAC spleens (see below), two pieces about the size of a grain of rice (about 20 mm^3) were randomly cut from the inside of each spleen. Samples from each spleen were suspended in 400 µl RAV1 buffer (NucleoSpin® 96 RNA Kit) and in 750 µl TRIzol® Reagent (Invitrogen, Darmstadt, Germany), respectively. Three 3 mm steel

beads were used for homogenization in a TissueLyser (Qiagen) for 2 min at 30 Hz. Afterwards, samples were centrifuged for 1 min at maximum speed in a microcentrifuge. From 140 µl supernatant of the homogenate, viral RNA was extracted using the QIAamp® Viral RNA Mini Kit. For the TRIzol® extraction 200 µl chloroform was added to the homogenate, mixed, incubated for 10 min at room temperature and centrifuged (10 min at maximum speed in a microcentrifuge cooled to 4 °C). The aqueous phase (up to 500 µl) was mixed with 600 µl ethanol. The lysate was then transferred to a RNeasy Mini column (Qiagen) and total RNA was extracted further according to the manufacturer's instructions.
For the blood-cell binding experiment, RNA was extracted from 100 µl of each sample by extraction robot (MICROLAB® STAR) with the NucleoSpin® 96 RNA kit and was eluted in 100 µl.

6.2. Detection of BTV RNA

Two different commercial RT-qPCR kits were used to detect BTV RNA: the iScript™ One-Step RT-PCR Kit (BIO-RAD, Hercules, CA, USA) and the Ambion AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Carlsbad, CA, USA). The iScript™ Kit was used as previously described (Hoffmann *et al*. 2006, Toussaint *et al*. 2007, Hoffmann *et al*. 2009c); the RT-qPCR thermal profile for the AgPath-ID™ Kit was 10 min at 45 °C and 10 min at 95 °C, followed by 42 cycles of 15 sec at 95 °C, 20 sec at 56 °C, and 30 sec at 72 °C in a Mx3005P® QPCR system (Stratagene, La Jolla, CA, USA). A total of 5 µl of eluate was used for RT-qPCR.
The group-specific "pan-BTV" assay detects the 24 BTV serotypes established at the time of the study by amplification of a highly conserved sequence on genome segment 5 (Toussaint *et al*. 2007), while the BTV-8 assay is directed to BTV genome segment 2, which encodes the highly variable serotype-specific outer shell protein VP2 (Hoffmann *et al*. 2009c).
Culicoides batches were analysed with the AgPath-ID™ Kit. RNA was amplified in a duplex RT-qPCR combining the detection of all confirmed 24 BTV serotypes ("pan-BTV" assay, Toussaint *et al*. 2007) and, as an internal control, the detection of all members of the genus *Culicoides* ("pan-Culicoides" assay, Hoffmann *et al*. 2009a). A total of 5 µl of eluate was used for RT-qPCR.
Blood samples of the cross-sectional study were analysed by using the iScript™ Kit and the group-specific "pan-BTV" assay.

All samples obtained from the animal experiment were tested with the "pan-BTV" and BTV-8 assay on the same PCR plate for cross-confirmation using the iScript™ Kit and the AgPath-ID™ Kit.

In vitro transcribed segment 5 standard RNA was serially diluted tenfold in RNA-safe buffer (Hoffmann *et al*. 2009c, Hoffmann, unpublished) and used for absolute quantification by external calibration. The resulting standard curves in all RT-qPCR runs were highly correlated (R^2 consistently >0.99) and demonstrated good amplification efficiency (slopes between -3.43 and -3.20) (data not shown). For all samples, BTV RNA quantification assays performed with the AgPath-ID™ Kit and the iScript™ Kit yielded very similar results (data not shown).

BTV RNA isolated from spleens with the QIAamp® Viral RNA Mini Kit was analysed with the "pan-BTV" segment 5 assay (Toussaint *et al*. 2007), the BTV-8 segment 2 assay (Hoffmann *et al*. 2009c), the commercially available group-specific Virotype® BTV Kit (Labor Diagnostik Leipzig, Leipzig, Germany; segment 10), and the group-specific cador BTV RT-PCR Kit (Qiagen; segment 1). RNA isolated with TRIzol® Reagent was tested with the "pan-BTV" and the BTV-8 assay using the AgPath-ID™ Kit as described earlier. An absolute quantification assay of the latter extracts was conducted with the *in vitro* transcribed and tenfold serially diluted standard RNA as previously described.

Samples of the blood-cell binding experiment were tested with the "pan-BTV" using the AgPath-ID™ Kit. The BTV RNA load was quantified as previously described.

6.3. Virus isolation in tissue culture and embryonated chicken eggs

Virus isolation from washed and ultrasonically disrupted (see below) blood cells was attempted for samples taken between 2 and 10 dpi, but no virus could be isolated (data not shown). RT-qPCR analysis of samples from the experimental infection and the blood-cell binding experiment revealed significantly higher amounts of BTV RNA in the plasma fraction than in cell pellets (see below). Therefore, virus isolation was repeated without prior washing. Whole blood samples were selected for lowest C_q value. Serum samples taken on the same day were negative in at least the SNT and cELISA. Virus isolation from twelve samples was attempted on Vero cells (6 to 10 dpi) and in ECE (2 to 6 dpi) by methods adapted from Clavijo *et al.* (2000). Vero cell cultures were inoculated with whole blood, blood cell pellets and plasma, while only whole blood was used for inoculation of 10-day-old ECE. Before inoculation, whole blood and blood cell pellets were ultrasonically disrupted on wet ice (full output, 90% duty cycle, 10 pulses, up to 3 times; Sonifier 450, Branson Ultrasonics Corp., Danbury, CT, USA) and centrifuged (1 min at maximum speed in a microcentrifuge). ECE

were then intravenously inoculated with 100 µl of supernatant and incubated for 7 days. The chicken embryos were harvested, and internal organs were homogenised in the TissueLyser (2 min at 30 Hz). Organ supernatants were used for virus isolation on 75% confluent Vero cell monolayers as described by Clavijo *et al.* (2000). After 5 days of incubation at 37 °C, 1 ml of cell culture supernatant was blindly passaged and incubated another 7 days. Organ supernatants and cell culture passages were tested for BTV RNA by RT-qPCR.

Virus isolation on Vero cells was also carried out with the supernatants of the homogenised spleens that were used for the inoculation of IFNAR$^{-/-}$ mice.

6.4. Virus isolation in IFNAR$^{-/-}$ mice

Virus isolation in IFNAR$^{-/-}$ mice (Calvo-Pinilla *et al*. 2009a) was attempted from whole blood taken between 2 and 6 dpi and spleens collected post-mortem at day 106.

For each SAC, whole blood samples from days 2 to 6 were mixed (250 µl each). For llama #5, only blood from 4 and 6 dpi was used (C_q values between 34.6 and 38.5; blood from llama #4 was negative in the RT-qPCR). The blood samples were processed as described above for ECE inoculation.

Spleen samples taken post-mortem had been stored at -80 °C. They were thawed, two rice grain-sized pieces were cut from each spleen as described above and suspended in 500 µl of Eagle's Minimal Essential Medium with antibiotics (MEM). The pieces were homogenised in the TissueLyser (2 min at 30 Hz) with one 5 mm steel bead and centrifuged for 1 min at maximum speed. The supernatants were pooled for each animal, and two more pools were prepared by mixing all alpaca and all llama samples, respectively. The samples were diluted with 1 ml of MEM and eight IFNAR$^{-/-}$ mice were then inoculated with 300 µl of sample each. The inoculation and further assessment of the mice was performed as described previously (Eschbaumer *et al*. 2010b).

7. Statistical analyses

The total number of female *Culicoides* caught on each of the farms were comparatively analysed by Kruskal-Wallis test (Kruskal and Wallis 1952) as well as paired two by two with Wilcoxon rank-sum tests (McCullagh and Nelder 1989, Hastie and Pregibon 1992, Venables and Ripley 2002) using Bonferroni correction to adjust the α-level. Furthermore, Wilcoxon rank-sum tests were used to determine significance between the total number of female *Culicoides* caught (i) indoor and outdoor on each farm, (ii) in each group (Obsoletus and Pulicaris complexes and "other" *Culicoides*) on each farm using Bonferroni correction, and

(iii) to compare daily *Culicoides* catches on farms B and C from July to November in the years 2008 and 2009.

The effects of the explanatory variables farm, temperature and humidity on the response variable (daily number of *Culicoides* caught in black-light traps) were modelled using multivariable Poisson regression in combination with (data not shown) and without an autoregressive model (time series analysis). See Table 7 for the definition of variables. All variables were analysed by automated stepwise regression using the Akaike information criterion (AIC; Sakamoto *et al*. 1986). Subsequently, the variables were tested for interaction. The same procedure (excluding the farm variable) was repeated separately for each farm.

For all analyses, only the number of *Culicoides* caught from July 2008 and June 2009 were considered, except for the comparison between the number of *Culicoides* collected on farms B and C from July to November in the years 2008 and 2009.

For all analyses, only the number of *Culicoides* caught from July 2008 and June 2009 were considered, except for the comparison between the number of *Culicoides* collected on farms B and C from July to November in the years 2008 and 2009.

For risk factor analysis, two statistical test series were set up in parallel: in the first series, all 91 farms and in the second, only the 43 farms with seropositive SAC were included in the analysis. In both cases, possible risk factors associated with BTV infection (dichotomous outcome variable) were analysed by Fisher exact tests (Fay 2010a) to determine their significance. For the age variable, the α-level was adjusted using Bonferroni correction. All explanatory variables were included in two multiple logistic regression models to analyse individual and herd variables separately. Variables were picked by automated stepwise backward and forward selection. The remaining variables were subsequently tested for interaction (McCullagh and Nelder 1989, Hastie and Pregibon 1992, Venables and Ripley 2002). For the definition of explanatory variables see Table 9.

Differences in the amount of viral RNA found in whole blood, serum and washed blood cell pellets in the experimental infection as well as in the blood-cell binding experiment were examined by Wilcoxon rank-sum tests. The relative sensitivity of the cELISA, DR ELISA and SNT to detect seroconversion after infection was compared in McNemar tests. The correlation between seroconversion and elimination of circulating viral RNA was examined with Cohen's kappa method.

The variances of antibody levels in SAC at different points in time (6 to 12 months) after vaccination were compared with two-sided *F*-tests. Results of both ELISA systems (VMRD ELISA and DR) (herd 1 and 4) were compared using a McNemar test (Agresti 1990, Fay

2010b). The level of agreement between the two tests was examined with Cohen's kappa method (Szklo and Nieto 2007).

All test results with *p*-values below an α-level of 0.05 were considered statistically significant. All statistical analysis was performed with the "R" software package (R Development Core Team 2010).

V. RESULTS

1. Entomological monitoring

Certain *Culicoides* spp. (Diptera, Ceratopogonidae) are vectors of various human and animal pathogens (Mellor *et al*. 2000). During the recent epizootic of BTV-8 in northern and central Europe, species belonging to the Obsoletus and Pulicaris complexes and *C. dewulfi* have been incriminated as the main vectors of this virus in ruminant herds (Meiswinkel *et al*. 2008, Hoffmann *et al*. 2009a, Eschbaumer *et al*. 2010a).

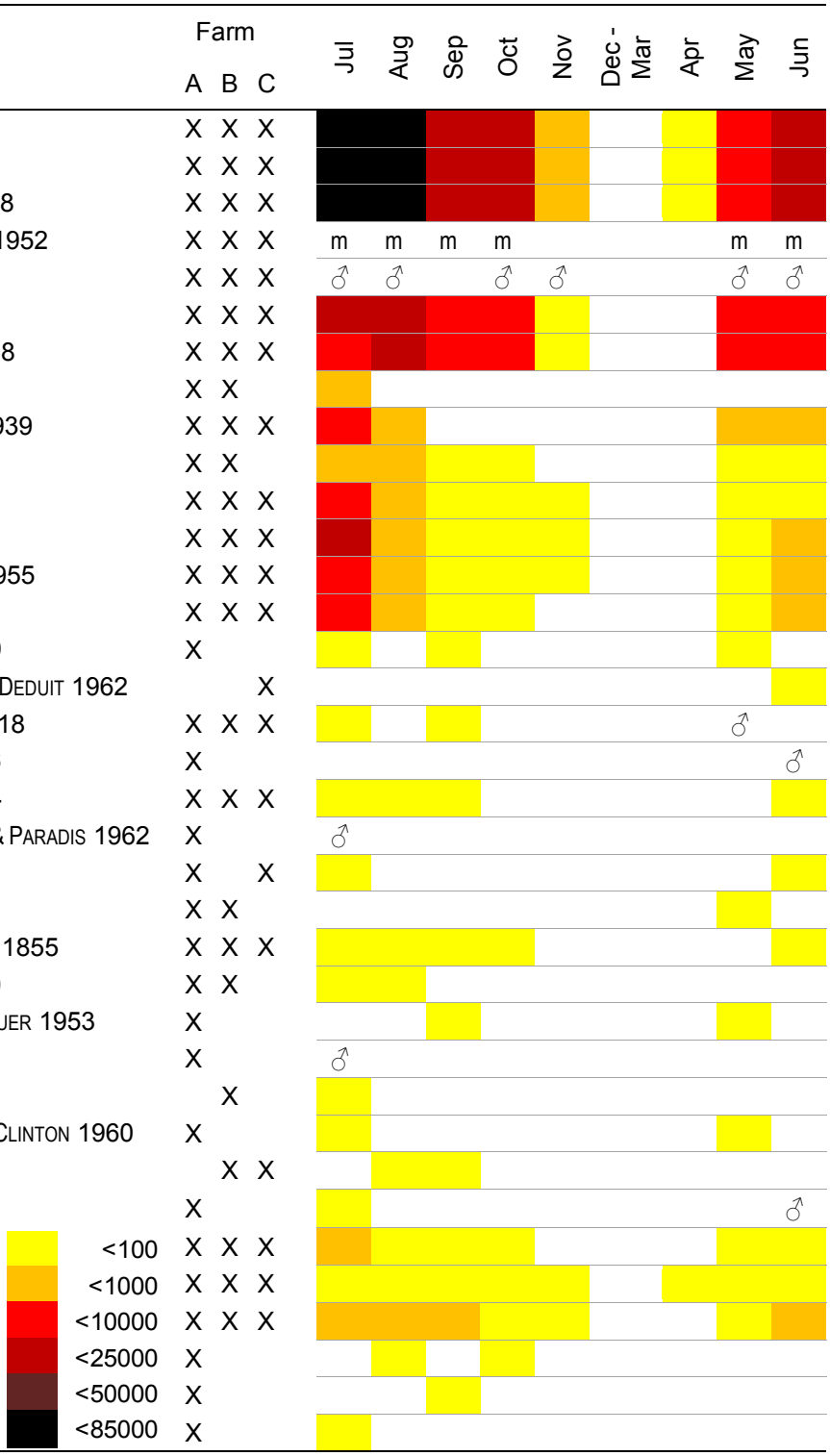
This is the first study investigating the fauna of *Culicoides* and other ceratopogonid genera on SAC farms. To this end, ceratopogonids were collected in black-light and emergence traps on three SAC farms in Germany, and selected *Culicoides* specimens were tested for BTV RNA. Furthermore, the factors location, temperature and humidity, which have been shown to influence the abundance of *Culicoides* and BTV infection rates on ruminant farms (Mellor *et al*. 2000, Purse *et al*. 2012), were analysed using multivariable Poisson regression.

1.1. Abundance of ceratopogonid females

About 266,100 female ceratopogonids were collected with the six black-light traps from July 2008 to June 2009; 98.8% belonged to species in the genus *Culicoides* (95.6% of about 281,400 from July 2008 to November 2009). The largest proportion belonged to species of the Obsoletus (77.4%) and Pulicaris (16.0%) complexes (Fig. 13 and Table 5), which have been suggested as the main potential BTV vectors in northern Europe (Hoffmann *et al*. 2009a). Details on proportions of *Culicoides* groups by trap location are shown in Table 6. Of the taxon *C. dewulfi*, which has recently been incriminated as novel potential vector for BTV (Meiswinkel *et al*. 2007, Meiswinkel *et al*. 2008), only a few males were collected on farm A (Table 5). The classical Afro-Asian BTV vector *C. imicola* KIEFFER 1913 (Meiswinkel *et al*. 2004) was not detected in any of the sorted specimens.

Table 5. Total monthly catches (indoor and outdoor) of females by *Culicoides* species (n=26) and other ceratopogonid genera in black-light traps on three SAC farms in Germany 2008/2009. The species *C. lupicaris* and *C. delta* are listed together as there is disagreement as to whether they can be morphologically separated from each other (Kiel *et al*. 2009, IAH 2009). (*Culicoides* tested with RT-qPCR are included in the table.)

Genus, complex or species[a]	Farm			Jul	Aug	Sep	Oct	Nov	Dec - Mar	Apr	May	Jun
	A	B	C									
Culicoides LATREILLE 1809	X	X	X									
Obsoletus complex:	X	X	X									
C. obsoletus s.s. MEIGEN 1818	X	X	X									
C. scoticus DOWNES & KETTLE 1952	X	X	X	m	m	m	m				m	m
C. chiopterus MEIGEN 1830	X	X	X	♂	♂		♂	♂			♂	♂
Pulicaris complex:	X	X	X									
C. pulicaris s.s. LINNAEUS 1758	X	X	X									
C. fagineus EDWARDS 1939	X	X										
C. lupicaris/delta EDWARDS 1939	X	X	X									
C. newsteadi AUSTEN 1921	X	X										
C. punctatus MEIGEN 1804	X	X	X									
Other *Culicoides* spp:	X	X	X									
C. achrayi KETTLE & LAWSON 1955	X	X	X									
C. albicans WINNERTZ 1852	X	X	X									
C. brunnicans EDWARDS 1939	X											
C. clastrieri CALLOT, KREMER & DEDUIT 1962			X									
C. circumscriptus KIEFFER 1918	X	X	X								♂	
C. dewulfi GOETGHEBUER 1936	X											♂
C. festivipennis KIEFFER 1914	X	X	X									
C. furcillatus CALLOT, KREMER & PARADIS 1962	X			♂								
C. grisescens EDWARDS 1939	X		X									
C. maritimus KIEFFER 1924	X	X										
C. minutissimus ZETTERSTEDT 1855	X	X	X									
C. nubeculosus MEIGEN 1830	X	X										
C. poperinghensis GOETGHEBUER 1953	X											
C. puncticollis BECKER 1903	X			♂								
C. riethi KIEFFER 1914		X										
C. segnis CAMPBELL & PELHAM-CLINTON 1960	X											
C. stigma MEIGEN 1818		X	X									
C. vexans STAEGER 1839	X											♂
Artrichopogon KIEFFER 1911	X	X	X									
Dasyhelea KIEFFER 1906	X	X	X									
Forcipomyia MEIGEN 1818	X	X	X									
Lasiohelea KIEFFER 1921	X											
Probezzia KIEFFER 1906	X											
Stilobezzia KIEFFER 1911	X											
Number of *Culicoides* spp.				21	13	13	13	5	0	1	14	15
Number of ceratopogonid genera				5	5	5	5	3	0	2	4	4



[a] www.culicoides.net; Werner and Kampen (2010); ♂, only males were caught from the species; m, only males were identifiable to the species *C. scoticus*.

Table 6. Incidence of females of the Obsoletus and Pulicaris complexes and other *Culicoides* spp. caught on three SAC farms in Germany from July 2008 to June 2009 by location of the black-light traps. On farms B and C, *C. obsoletus s.l.* were mainly caught indoor, while *C. pulicaris s.l.* predominated outdoor collections. In contrast, these proportions were similar on farm A. (The table includes *Culicoides* analysed by RT-qPCR.)

Farm	Trap location	Total no.	Proportion (%)		
			Obsoletus complex	Pulicaris complex	Other *Culicoides* spp.
A	Outdoor	178,478	75.7	16.3	8.0
	Indoor	73,934	80.2	16.1	3.7
B	Outdoor	2,684	73.4	22.6	4.0
	Indoor	4,347	95.7	3.3	1.0
C	Outdoor	1,384	70.7	20.2	9.1
	Indoor	2,048	88.3	8.2	3.6

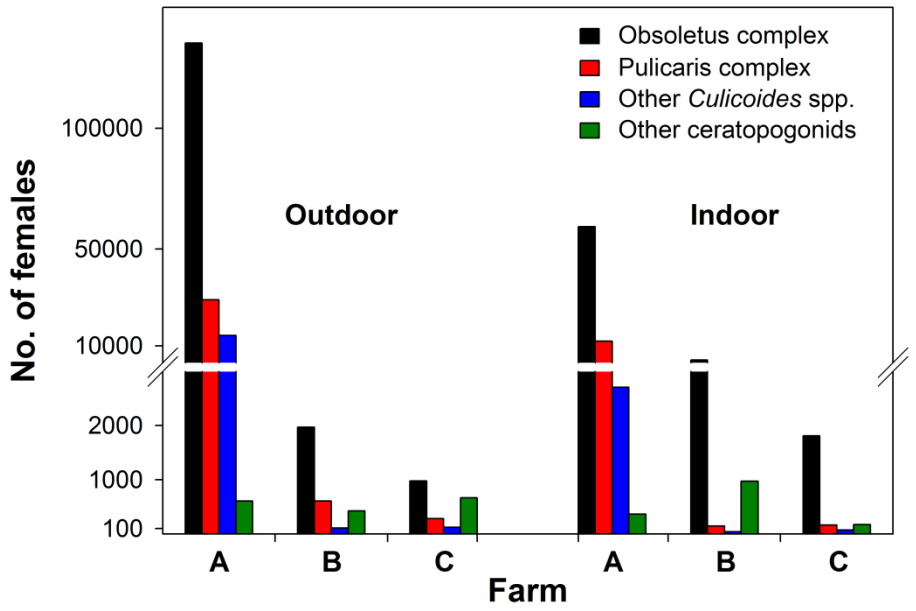


Fig. 13. Incidence of female *Culicoides* and other female ceratopogonids collected out- and indoor on three SAC farms from July 2008 to June 2009. Significantly ($p < 0.05$) more *Culicoides* were caught on the Hessian farm (A) (95.5%) than on the farms in Lower Saxony (B) and Bavaria (C), respectively. Species of the Obsoletus complex significantly predominated the specimens in indoor and outdoor collections on all farms, except outdoor on farm C where this difference was not significant. (*Culicoides* tested with RT-qPCR are included in the figure.)

Daily catches of female *Culicoides* varied from none in winter (December 2008 through March 2009) to up to 19,789 (maximum outdoor: 17,605; maximum indoor: 9,174) mostly *C. obsoletus s.l.* in both traps together on farm A at one day in July 2008. The number of *Culicoides* on each of the three farms peaked outdoor in July and indoor in August 2008 (see also Table 5). In 2009, most *Culicoides* were caught in July (farm B) or in June and August (farm C) (Fig. 14) both in out- and indoor traps. Significantly more female *Culicoides* were caught on farm A (96.0%) than on farms B (2.7%) and C (1.3%), but no significant difference was found between catches on farms B and C. Considering all three farms, the number of caught female *Culicoides* differed significantly.

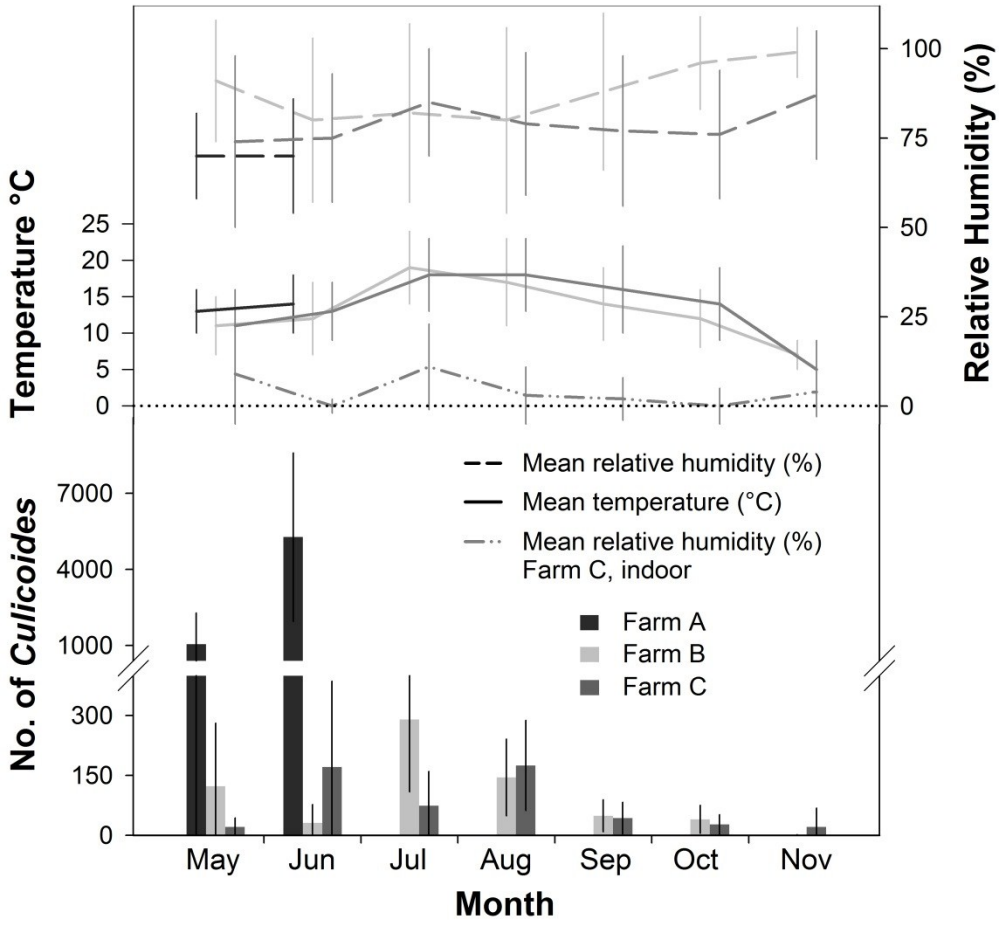


Fig. 14. Total number of female *Culicoides* caught outdoor and indoor versus temperature and relative humidity measured outdoor on three SAC farms in Germany in 2009. Whiskers indicate standard deviation. Temperature and humidity measured out- and indoor at the respective farm were very similar (data not shown), except at farm C where the humidity was considerably lower indoor than outdoor. The interaction of temperature and humidity significantly correlated with the daily number of *Culicoides*. Farm A was not included in the study after July 2009.

On farm A, the abundance of female *Culicoides* was higher outdoor (70.7%) than indoor, while on the other two farms this proportion was inversed: about 39% were caught outdoor (Fig. 13). A significant difference between outdoor and indoor collections was found for farm B but not for farms A and C. The proportion of ceratopogonids of other genera caught on farms A and C was higher outdoor (62.4% respectively 79.3%), but on farm B indoor collections (69.6%) exceeded the number of other ceratopogonids caught outdoor (Fig. 13). *C. obsoletus s.l.* were caught during the whole study period (not in winter), except outdoor on farms B and C in April 2009 (Table 5). Similarly, no *C. pulicaris s.l.* were caught from December 2008 to April 2009 in- and outdoor on any of the farms. Additionally, no *C. pulicaris s.l.* were collected outdoor in Oktober 2008 and June 2009 (farm B) or in November 2008 (farms B and C).

1.2. Abundance of ceratopogonid males

A total of 0.8% of about 268,300 ceratopogonids caught from July 2008 to June 2009 were males. The overall proportion of males was considerably lower in the genus *Culicoides* (0.5% of about 264,100) than in other genera (23.6% of 4207).

The number of Obsoletus males peaked between July and October 2008 and in May or June 2009, but their abundance varied considerably between trap locations and farms. *C. pulicaris s.l.* males were predominantly caught outdoor from July to September 2008 (farms A and B) and in June 2009 (farm A), but none was collected on farm C. Only single individuals were sporadically caught indoor between July and October 2008 and in June 2009.

The proportion of male *Culicoides* by trap location varied in each group on each farm from 0.0 to 5.6%. Similar to the collections of females on farms B and C (Fig. 13), a trend towards a higher proportion of *C. obsoletus s.l.* males indoor and *C. pulicaris s.l.* males outdoor was noted (Fig. 15). Furthermore, proportions were higher indoor on farms B and C (1.3 vs. 5%), while this was very similar outdoor and indoor on farm A (about 0.4%).

Monthly proportions of *Culicoides* males of both complexes were between 0.0 and 1.6% on farm A. On the other two farms, the proportion of Obsoletus males mostly varied from 0.0 to 13.5% indoor and from 0 to 6.5% outdoor. Exceptions were found outdoor on farm B in November 2008 (20.0%, 1 of 5), indoor on farms B and C in April (50%, n=2 and 4) and on farm C in May 2009 (36.1%, 44 of 122) when only a few midges were caught.

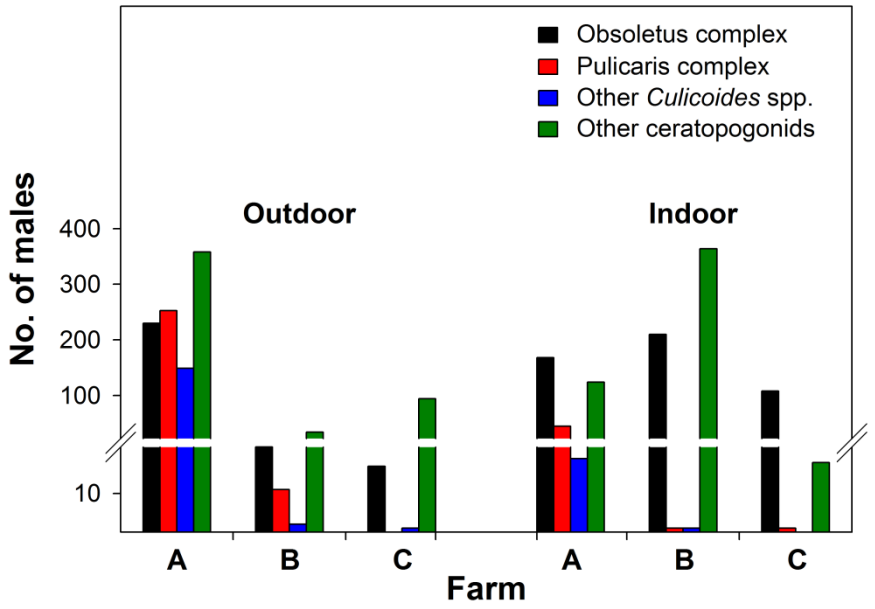


Fig. 15. Incidence of male *Culicoides* and other male ceratopogonids collected out- and indoor on three SAC farms from July 2008 to June 2009. The proportional distribution within the *Culicoides* groups was generally similar to that of females. However, the proportion of other ceratopogonids was considerably higher for males than for females. (See Fig. 13 for comparison with females.)

1.3. Annual deviations

Annual deviations in ceratopogonid collections (July to November 2009 vs. 2008) predominantly occurred in the number of *Culicoides* females on farm B and the number of other ceratopogonid females on farm C. On farm B, *C. obsoletus s.l.* and *C. pulicaris s.l.* females occurred three respectively five times less frequently outdoor and *C. pulicaris s.l.* females three times less frequently indoor in 2009 (data not shown) than in 2008. However, the difference in the number of *Culicoides* caught in the two consecutive years was not significant. On farm C, the number of females of other ceratopogonid genera was ten times higher outdoor and seven times higher indoor in 2009 compared to 2008.

1.4. Phenology of ceratopogonids

The highest diversity of genera (7 of 7) and *Culicoides* spp. (23 of 26) was collected on farm A, while on farms B and C midge collections consisted of 4 genera and 17 and 14 *Culicoides* spp., respectively (Table 5). The diversity of *Culicoides* spp. and other genera of ceratopogonids caught outdoor and indoor on all three farms peaked in July in both study years (Fig. 16 and Table 5; data of 2009 not shown). On farm level, the same was found for

farms A and B in both years, except in July and August 2008 on farm B when the number of species and genera was equal. In contrast, on farm C the diversity peaked outdoor in July and September and indoor in June, August and October 2008, while in 2009, peaks occurred outdoor in June, August and October and inside in June and July.

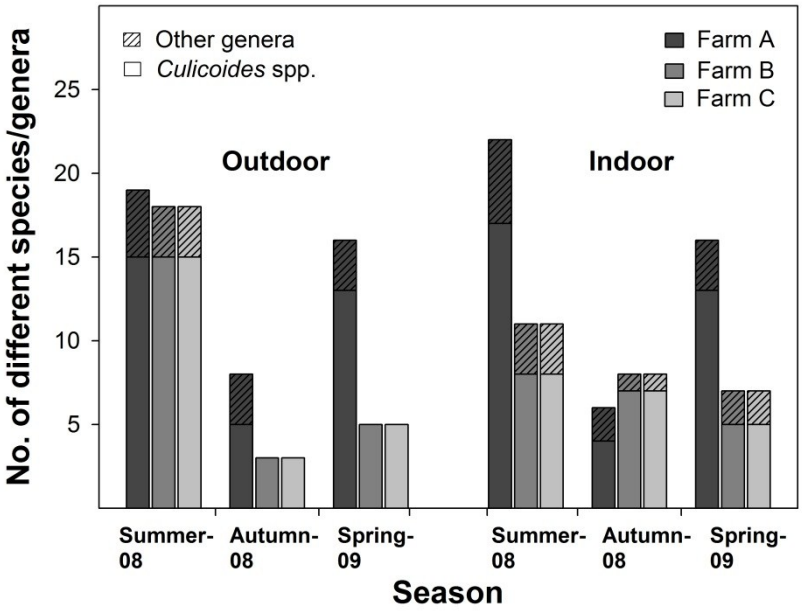


Fig. 16. Phenology (females and males) of *Culicoides* spp. and other genera of ceratopogonids caught on three SAC farms from July 2008 to June 2009. The diversity of species of the genus *Culicoides* and other genera of ceratopogonids peaked in summer out- and indoor on all farms.

1.5. Association between daily number of *Culicoides* and farm, temperature and humidity

The daily number of *Culicoides*, temperature and relative humidity varied considerably during the catching periods (Fig. 14). The highest abundance was found at temperatures of around 20°C and a humidity of around 75%. In general, low temperatures had a negative effect on the number of midges. A few individuals were caught at temperatures as low as 6 °C.

An overview of factors that significantly influenced the number of *Culicoides* midges on all farms is given in Table 7. The number of female *Culicoides* midges was predominantly influenced by the farm variable (Table 7 and Fig. 14) and depended on the interaction of temperature and humidity (Table 7). This interaction term had a positive effect on the daily number of *Culicoides* comparing data from outdoor and indoor weather stations separately, but a negative effect comparing indoor collections with outdoor weather data. Analysing interaction terms by farm, results were contradictory and no consistent pattern was identified

(data not shown). However, an interaction between the temperature and humidity variables was generally present, except indoor on farm B. Poisson regression analysis combined with an autoregressive model revealed very similar results compared to the analysis done without this model. However, days on which no *Culicoides* were caught in the traps were not considered in the model. Therefore, data of the extended model are not presented. A general measurement error must be assumed for the humidity measured indoor at farm C as it was extremely low (0 to 11%) compared to the values measured outdoor. On the other hand, the dynamics of the humidity over the measurement period was very similar to that measured with the weather station outdoor (Fig. 14).

Table 7. Summary of the results of Poisson regression analyses showing significant ($p < 0.05$) covariates in the model.

Location of trap	Location of weather station	Variable	Category	IRR	$CI_{95\%}$ for IRR		*P*
					Lower	Upper	
Outdoor	Outdoor	Farm	A (Intercept[a])	4.02e+02	2.61e+02	6.21e+02	s
			B	0.0014	0.0013	0.0015	s
			C	0.0033	0.0031	0.0035	s
		Temp		0.6903	0.6678	0.7134	s
		Hum		1.0014	0.9956	1.0072	ns
		Temp:Hum		1.0070	1.0065	1.0074	s
Indoor	Indoor	Farm	A (Intercept[a])	1.72e+03	1.40e+03	2.11e+03	s
			B	0.0357	0.0340	0.0375	s
			C	0.0065	0.0057	0.0074	s
		Temp		1.0991	1.0878	1.1105	s
		Hum		0.9354	0.9326	0.9382	s
		Temp:Hum		1.0027	1.0026	1.0029	s
Indoor	Outdoor	Farm	A (Intercept[a])	0.4048	0.2622	0.6259	s
			B	0.1010	0.0961	0.1061	s
			C	0.0336	0.0318	0.0354	s
		Temp		1.8395	1.7912	1.8888	s
		Hum		1.0500	1.0439	1.0561	s
		Temp:Hum		0.9953	0.9949	0.9957	s

IRR, Incidence rate ratio; Temp, temperature (°C); Hum, relative humidity (%); Temp:Hum, IRR analysing interaction of both variables; [a] Reference group; [s], significant; [ns], not significant

1.6. Virological results

A total of 11.1% (7,177 of 64,693) of all counted (2.7% of estimated 270,543) *Culicoides* were tested for BTV RNA. None of the tested *Culicoides* batches was positive for BTV RNA.

1.7. Ceratopogonids in emergence traps

The number of caught ceratopogonids was considerably lower in emergence traps than in black-light traps (Table 5, Fig. 13 and Fig. 17). The abundance of *Culicoides* (159 vs. 21) and other ceratopogonid (144 vs. 4) females was considerably higher in the 'faeces trap' compared to the 'pasture trap'. About half (52.5%; 159 of 303) of the ceratopogonid females caught in the faeces trap belonged to the genus *Culicoides*, while this proportion was about one third higher (84.0%, 21 of 25) in the pasture trap.

Monthly collections of female *Culicoides* peaked twice: in May and in August 2009 (Fig. 17), but no ceratopogonids were caught in September 2009. In the pasture trap, *Culicoides* were only caught in May, except for *C. minutissimus*, which was caught once in August. Females of the Obsoletus complex (90 of 94), *C. achrayi* (all 36) and *C. punctoicollis* (n=1) were caught predominantly or exclusively in the faeces trap. In contrast, *C. albicans* were primarily caught in the pasture trap (16 of 18).

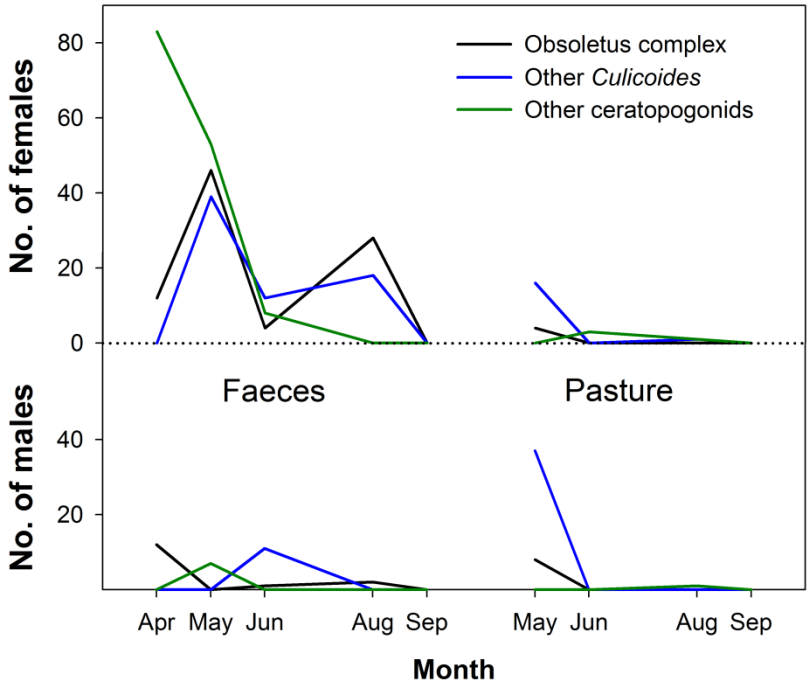


Fig. 17. Ceratopogonid females and males caught in emergence traps set up over a pile of llama faeces and on a dung-free area on the same pasture on a SAC farm in Hesse, Germany, during the vegetative period - from April to September 2009. No ceratopogonids were caught in September 2009.

About two thirds (n=71) of all male *Culicoides* were caught in the pasture trap. Inversely, considering *C. obsoletus s.l.* males separately about two thirds (n=23) were collected in the faeces trap. Comparing proportions of Obsoletus males and females caught in emergence traps with those collected in the outdoor black-light trap on farm A, the proportion of males was considerably higher in the faeces trap (14.3% of 105) than in the black-light trap (0.2% of about 138,400). In the pasture trap, *Culicoides* males (68.2% of 66) and Obsoletus complex males (66.7% of 12) even predominated the collections (Fig. 17).

2. Cross-sectional study

The potentially devastating consequences of BT (Conraths *et al*. 2009), the increasing popularity of SAC (Schulz *et al*., manuscript in preparation) together with the recent BTV-related fatalities (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b) prompted the investigation of BTV infection in SAC. Therefore, a large-scale serological and virological survey was conducted. Blood samples were tested for BTV antibodies by ELISA, and all seropositive samples for BTV RNA with RT-qPCR. Since none of the serological systems were validated for the detection of BTV antibodies in SAC at the time, five different commercially available ELISAs were compared with some sera of unvaccinated SAC. All serological results of this study were based on the results obtained by PrioCHECK® ELISA.
Data on species, sex, age, parentage, and BTV-related disease, fatalities and abortions in SAC in Germany was collected by questionnaire. Furthermore, factors influencing BTV infection in ruminant husbandry such as the use of insecticides, housing or animal movement were also evaluated for SAC. Various statistical analyses were conducted to identify risk factors for BTV infection in SAC.

2.1.1. Study population

The response rate of the telephone interview was 100% (n=227). The majority of SAC owners (91 of 97, 94%) who generally kept at least 8 unvaccinated animals agreed to participate in the cross-sectional study. Of these, 51 (56%) were members of the NWK e.V. Of 2601 SAC on 91 farms, 1742 (67%) were tested for BTV antibodies. The species, sex and age ratios as well as the regional distribution of the 91 tested herds were representative for almost 4,000 SAC kept on the 227 contacted farms (data not shown). Information on the number of SAC tested per herd is given in Table 3. Unfortunately, a high proportion of owners (36 of 91, 39.6%) only allowed sampling in a lower number of animals than required when assuming a

5% prevalence (Table 3). For the number of individuals or herds included in statistical analyses for each variable see Table 9.

2.1.2. Prevalence of BTV infection

Seropositive animals were found in all SAC species and breeds, except the purebred vicuñas (other animals of the same herd were also negative). A total of 249 animals (27.6%, 95% confidence interval [95% CI]: 24.7–30.7%) on 43 farms (47.3%) with 901 tested SAC were BTV-seropositive. Considering all 91 herds, the seroprevalence was 14.3% (Table 8; Table 1 in Appendix 4). Detailed information about seroprevalences by species, sex and age groups is given in Table 9 and in Table 2 in Appendix 4.

BTV-8 seroprevalence within infected SAC herds varied between 2 and 100%. The highest regional and within-herd seroprevalence was found in Western Germany. For details on seroprevalence by region see Fig. 18, Table 8 & Table **9** and Table 1 in Appendix 4.

No BTV RNA was detected in any of the BTV antibody-positive animals (95% CI: 0–0.01%; 0 of 249) using RT-qPCR.

Table 8. BTV-8 seroprevalence in SAC (n=1742) and SAC herds (n=91), and median within-herd seroprevalence (n=43) by region in Germany 2008/2009. (See also Table 1 in Appendix 4 for seroprevalence by federal state in Germany.)

Region (federal state)	Individual SAC			Herds			Positive herds
	No. of samples	No. positive	% (95% CI)	No. of samples	No. positive	% (95% CI)	Median (25%-75% Q)
West (North Rhine-Westphalia, Hesse, Rhineland-Palatinate, Saarland)	391	167	42.7 (37.8-47.8)	23	18	78.3 (5.6-9.3)	56 (27-80)
Northern-coastal (Mecklenburg-Western Pomerania, Lower Saxony, Schleswig-Holstein)	223	25	11.2 (7.4-1.6)	12	9	75.0 (42.8-94.5)	15 (8-26)
South (Baden-Wuerttemberg, Bavaria)	751	54	7.2 (5.4-9.3)	37	15	40.5 (24.8-57.9)	14 (6-33)
East (Brandenburg, Saxony, Saxony-Anhalt, Thuringia)	377	3	0.8 (0.2-2.3)	19	1	5.3 0.1-26.0)	33[a] (-)
Total	1742	249	14.3 (12.7-16.0)	91	43	47.3 (36.7-58.0)	26 (12-51)

95% CI, 95% confidence interval; Q, quartiles, [a] seroprevalence in one positive herd in Saxony-Anhalt

Table 9. Definition of explanatory variables, number of individuals or herds included in statistical analyses for each variable, and BTV seroprevalence in SAC in Germany 2008/2009 by variable level.

Unit of observation	Variable	Level	All 91 herds			43 positive herds	
			No. of samples	No. positive	% positive	No. of samples	% positive
Individual	Location*	East	727	10	1.4	85	11.8
		West	1015	239	23.5	816	29.3
	Age	Yearling	211	18	8.5	547	32.9
		Cria	406	51	12.6	98	18.4
		Adult	1064	180	16.9	210	24.3
	Species	Alpaca	1249	146	11.7	540	27.0
		Llama	479	95	19.8	350	27.1
	Sex	Female	1011	150	14.8	508	29.5
		Male	611	91	14.9	307	29.6
Herd	Location*	East	37	4	10.8		
		West	54	39	72.2		
	Ruminants	Ruminants	27	11	40.7		
		No ruminants	64	32	50.0		
	Housing	Always access to a shelter	89	41	47.2		
		Only stabled at night	2	2	50.0		

* Western or eastern half of Germany

2.1.1. Questionnaire

2.1.1.1. Natural BTV infection in SAC

Fatalities related to BTV infection were recorded for three SAC: one 3-year-old llama mare in Hesse (February 2008), one adult alpaca mare in Thuringia (December 2008) and one 7-month-old alpaca in Lower Saxony (January 2009). Clinical disease and pathological findings described in the post-mortem report of the llama were similar to observations for a perished alpaca by Henrich *et al.* (2007). BTV RNA and antibodies were present in the post-mortem samples, and BTV infection was named as the cause of illness in the post-mortem report. However, sepsis was suspected as the final cause of death. For the two alpacas, BTV infection was reported as a secondary pathological finding. The presence of BTV-8 RNA in organ

material was confirmed by the German national reference laboratory for bluetongue disease (Friedrich-Loeffler-Institut, Insel Riems; C_q values of 29.0 to 33.5).

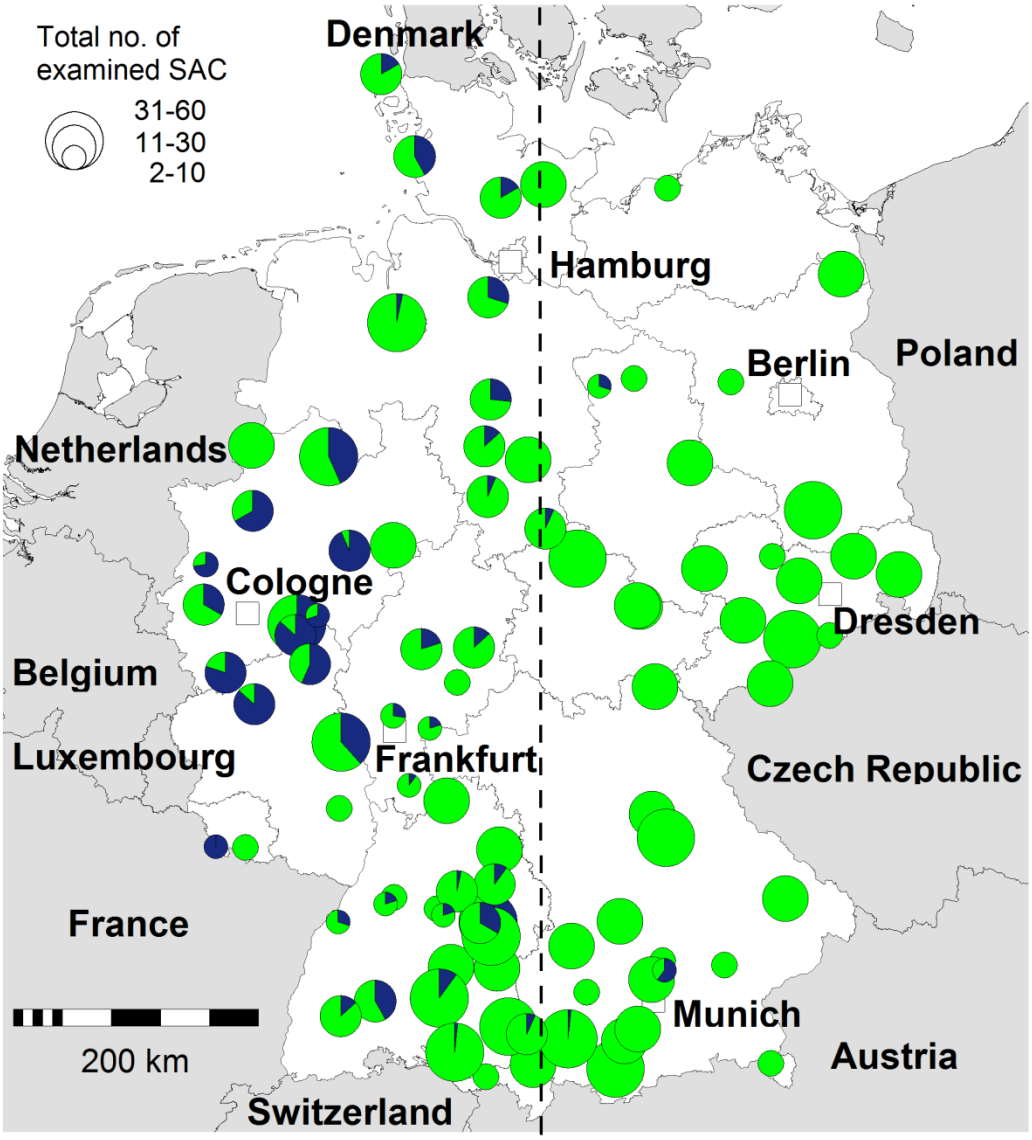


Fig. 18. BTV-8 seroprevalence in SAC 2008/2009. Map of Germany showing the 91 participating farms. Dot sizes indicate the proportion of tested SAC per herd; dark blue and green wedges indicate the proportion of seropositive and seronegative SAC on a farm, respectively. Seropositive SAC were found in 47.3% (43 of 91) of the herds. Altogether, 91% (39 of 43) of the positive farms were located in the western half of Germany (left of dotted line).

2.1.1.2. Animal movements

From 2006 to spring 2009, 24 seropositive SAC had been traded within or imported to Germany. For 5 of 13 SAC that had been traded within Germany a BTV infection before their purchase can be assumed as the receiving farm was located in Bavaria, a region where only a

few BTV cases occurred in ruminants (Conraths *et al*. 2009) or SAC (Fig. 18). In May 2008, these llamas were purchased from Northern Germany where a high BTV prevalence was recorded (Conraths *et al*. 2009). Two llamas that had been kept on the receiving farm before were seronegative. Of the eleven imported animals, eight had been purchased from Switzerland before May 2007 and three from Chile in 2006, at a time when both countries were considered free of BTV (Pinto *et al*. 2008, Zanolari *et al*. 2010b).

2.1.1.3. Association between explanatory variables and BTV infection

Table 10 provides an overview of risk factors associated with BTV infection in SAC. In univariate analysis the explanatory variables location, age and species were significant, while in the multivariate logistic regression model only location and age remained significant risk factors for BTV infection (Table 10).

Table 10. Logistic model of risk factors for BTV infection in SAC, Germany, 2008/2009.

Unit of observation	Variable	Level	Odds ratio (95% CI)	
			All 91 herds	43 seropositive herds
Individual	Location*	East	Reference	Reference
		West	23.9 (13.28 - 48.59) [s]	3.64 (1.93 - 7.63) [s]
	Age	Yearling	Reference	Reference
		Cria	1.62 (0.92 - 2.98)	1.55 (0.86 – 2.89)
		Adult	2.33 (1.41 – 4.06) [s]	2.39 (1.42 - 4.23) [s]
		Cria	Reference	Reference
		Adult	1.43 (1.01 – 2.06) [s]	1.54 (1.08 – 2.24) [s]
Herd	Location*	East	Reference	
		West	21.45 (7.12 - 81.61) [s]	

95% CI, 95% confidence interval; [*] Western or eastern half of Germany; [s], significant ($p < 0.05$)

The odds ratio (OR), 95% CI (of the OR) and *p*-values obtained for the age variable by separate univariate analyses of all herds or the seropositive herds were all very similar (data not shown). When analysing interaction terms, only the location remained significant in the model (all herds: OR=29.5, 95% CI 14.1–75.5). The same was found when analysing the seropositive herds separately, but the OR was considerably lower (OR=5.0, 95% CI 2.28–13.2).

3. Animal experiment

The unknown role of SAC in BTV epidemiology (Mattson 1994) together with the reported fatalities (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b) prompted further investigations of BTV infection in SAC.

To this end, a small-scale animal experiment has been conducted to collect data on the susceptibility of SAC to BTV-8 infection and reference material for the validation of serological (ELISA and SNT) and virological assays (RT-qPCR and virus isolation).

3.1. Clinical and haematological parameters

Before the experimental BTV infection, the SAC were healthy, and their clinical and haematological parameters were normal as described by Fowler (1998a) and Hengrave Burri *et al.* (2005). After infection, fever (>39 °C) was never recorded until the end of the experiment. Mild conjunctivitis was seen in all animals around 3 dpi. All animals showed extended recumbency on 3 and 4 dpi. One llama displayed additional signs of discomfort with reduced attention and activity, floppy ears, a bent spine and reduced feed intake from 5 to 6 dpi. Another llama showed the same signs except the bent spine up to 5 dpi and had low-grade lung sounds from 5 to 8 dpi that could be exacerbated in a stress test (holding the nostrils closed until the animal shows decisive resistance). One alpaca showed extended recumbency until 7 dpi. From 2 to 6 dpi, leukocyte counts were considerably reduced, but were back at their initial values by 20 dpi (Fig. 19).

3.1. Serology

Dynamics of antibody development are summarized in Fig. 20 to Fig. 22. Table 11 gives an overview of the time of seroconversion in the three different test systems. The ELISAs, especially the DR ELISA, detected seroconversion earlier than the SNT. This difference, however, was not statistically significant.

After the initial increase, antibody levels in the cELISA and antibody titres in the SNT remained stable. Conversely, antibody levels in the DR ELISA showed a remarkable decline between 20 to 35 dpi, but then remained stable after a second peak 62 dpi (Fig. 20 to Fig. 22).

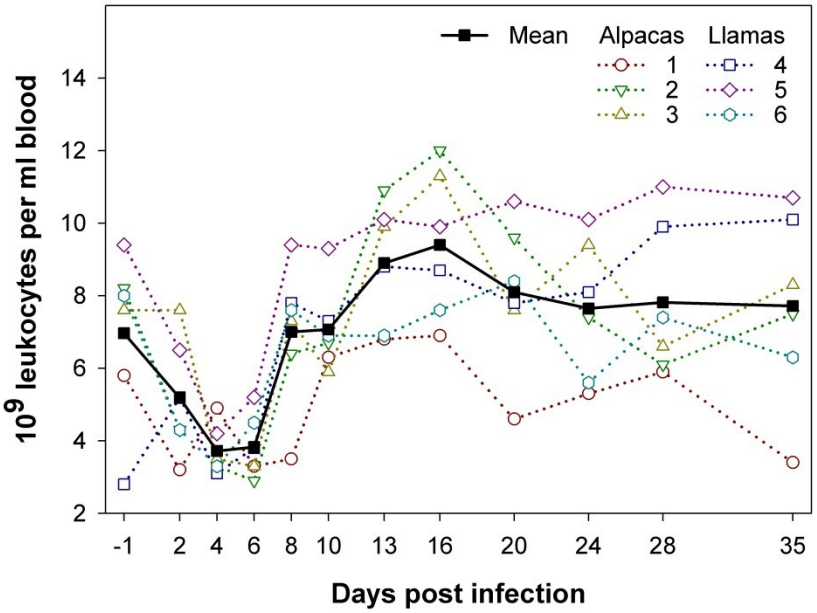


Fig. 19. Leukocyte counts in SAC blood after experimental BTV-8 infection.

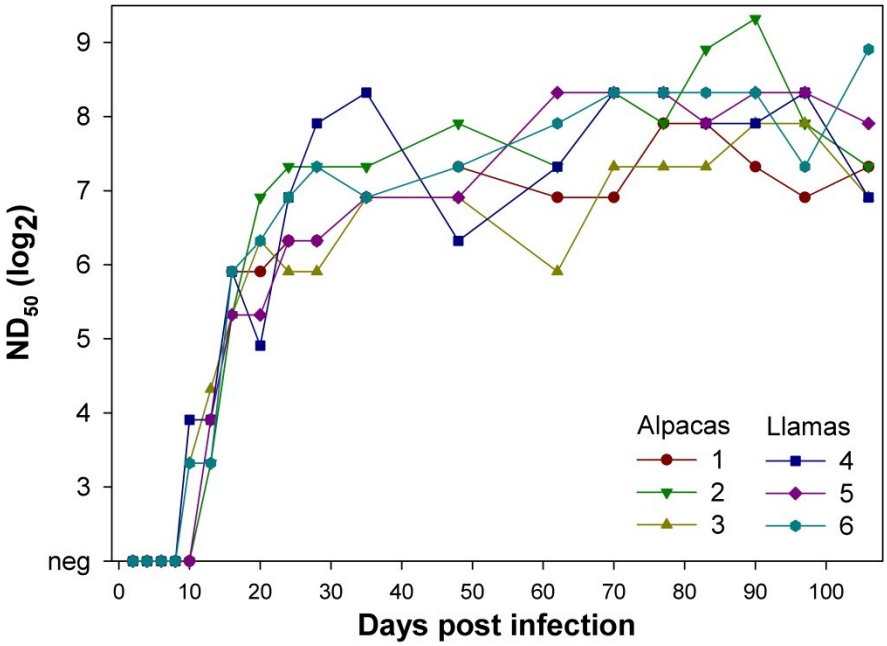


Fig. 20. Neutralising antibodies in SAC after experimental BTV-8 infection.

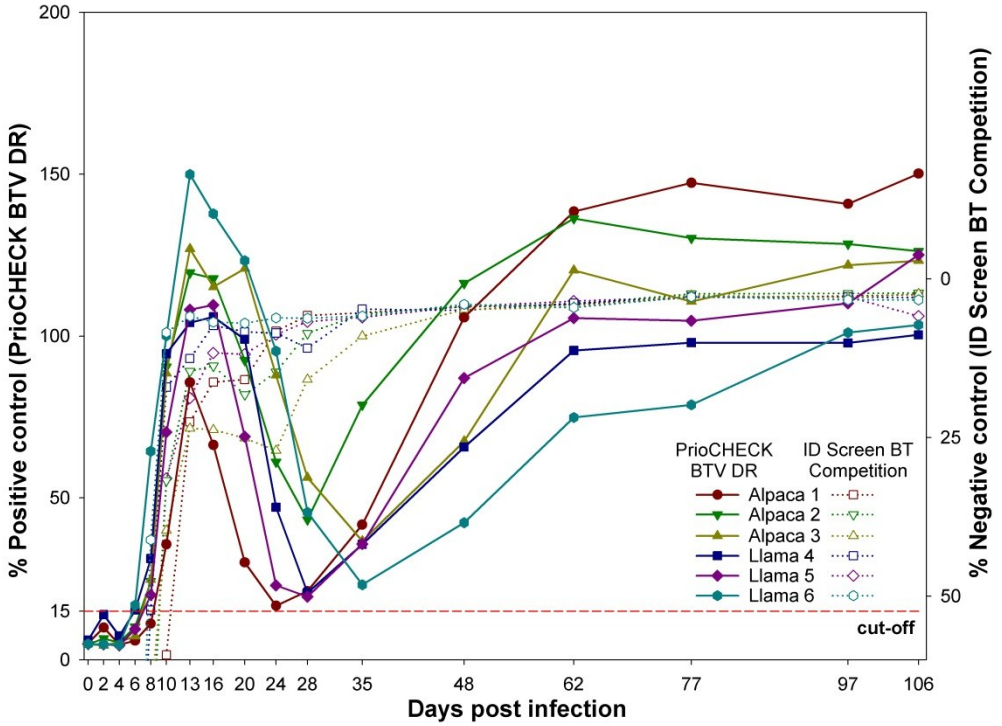


Fig. 21. Antibody response after BTV-8 infection of SAC in the PrioCHECK® BTV DR and the ID Screen® BT Competition ELISA.

Table 11. Seroconversion after experimental BTV-8 infection of SAC (n=6) as measured in three different test systems.

	Number of positive animals		
dpi	DR ELISA[a]	cELISA[b]	SNT
0 - 4	0	0	0
6	**2**	0	0
8	**5**	**1**	0
10	**6**	**5**	**3**
13 - 106	**6**	**6**	**6**

[a] PrioCHECK®; [b] ID Screen® BT Competition;

[c] Serum neutralisation test; dpi, days post-infection

3.1. Detection of BTV RNA in blood samples

Only negligible differences were found between results obtained from RNA extracted by robot (NucleoSpin® 96 RNA Kit) or manually (QIAamp® Viral RNA Mini Kit). There was good agreement between the "pan-BTV" assay, the BTV-8 assay and the two commercial BTV RT-qPCR kits (data not shown). Based on *in vitro* transcribed RNA standards, the limit

of detection of BTV RNA for the pan-BTV S5 assay using the iScript™ or AgPath-ID™ One-step RT-PCR kits was less than 10 copies per reaction.

Low amounts of BTV-8 RNA were detected in all SAC with peak values at 2 and 8 dpi. Viral genome was found in some SAC for up to 5 weeks, all animals were consistently positive until day 16 (Fig. 22). BTV-8 was first detected 2 dpi or 6 dpi in whole blood and 2 dpi in the serum of all animals. The highest amounts of BTV RNA were detected 8 dpi in whole blood (C_q values 31.2 to 34.1; 4.2 x 10^3 to 9.9 x 10^4 copies/ml) and in serum (C_q values 32.5 to 35.3; 3.7 x 10^3 to 6.2 x 10^4 copies/ml) of five SAC. Llama #6 showed lowest C_q values 6 dpi in whole blood (C_q 34.2; 1.7 x 10^4 copies/ml) and 10 dpi in serum (C_q 34.2; 2.6 x 10^4 copies/ml). The amount of viral RNA in whole blood and serum was significantly higher than in cell pellets for the days 2, 6 and 8. No significant differences were found for 4 and 10 dpi, and between serum and whole blood from 2 to 20 dpi. The tremendous decrease in the amount of detectable BTV RNA seen in all blood fractions after 8 dpi highly correlated with seroconversion (Fig. 22).

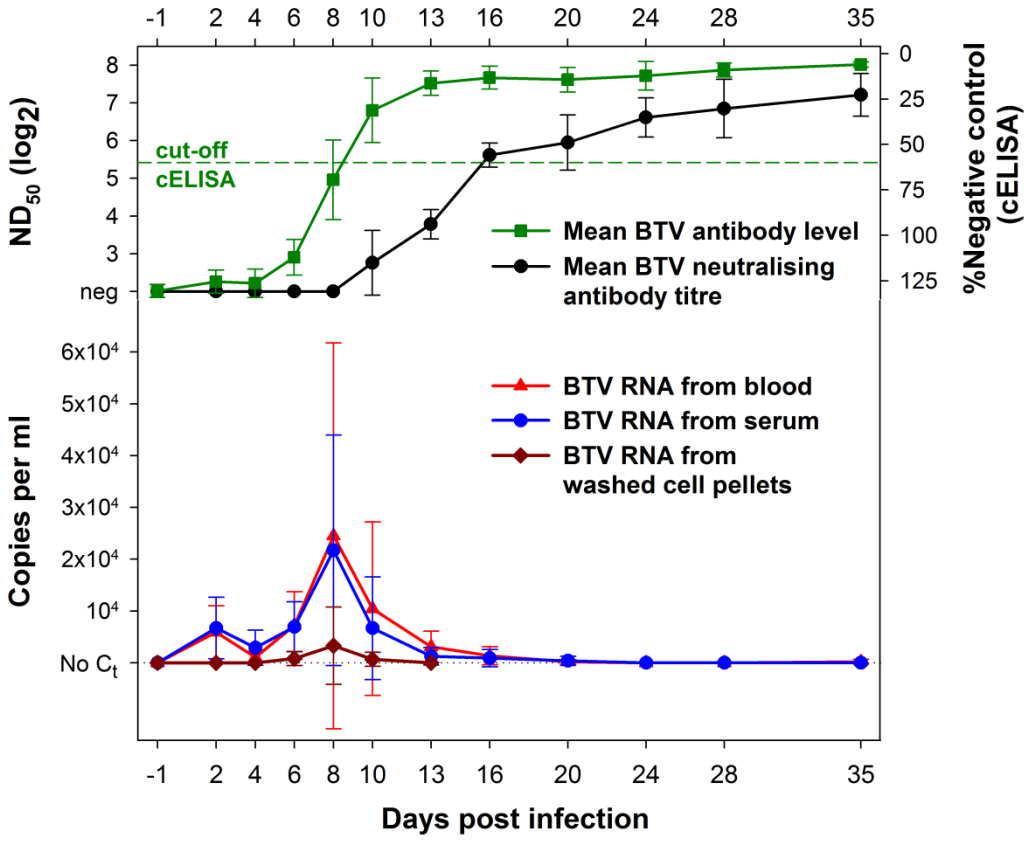


Fig. 22. Mean RT-qPCR results (absolute quantification of BTV genome) for whole blood, serum and washed blood cells set against serology after experimental BTV-8 infection of SAC. (Error bars indicate standard deviation.) Low amounts of BTV-8 RNA were detected in individual SAC for up to 5 weeks, but it was largely eliminated from blood after seroconversion.

Using Fleiss' criteria (1981), the point estimate of kappa suggests an excellent agreement with seroconversion measured by SNT or cELISA (0.82), but only a fair to good agreement for the DR ELISA (0.63). In all animals, low amounts of BTV RNA were detected in whole blood until 16 to 35 dpi and in serum until 20 to 28 dpi. In washed blood cell samples, RNA was detected intermittently from2 to 10 dpi in all alpacas and one llama (C_q values over 34.8).

3.2. Post-mortem examination and detection of BTV RNA

Post-mortem examination was conducted 71 to 78 days after the last BTV RNA detection in whole blood and serum. No lesions typical for BT were found in any animal. Surprisingly, spleen samples of all animals were positive for BTV RNA. C_q values ranged from 30.7 to 36.9 in the "pan-BTV" and BTV-8 assays. In the commercially available BTV-specific RT-qPCR kits (segment 1 and 10), C_q values were higher or over 42 cycles, but showed a trend similar to the C_q values of the custom assays.

3.3. Virus isolation

Virus could never be isolated by cell culture or ECE. To detect replication-competent virus with higher sensitivity, IFNAR$^{-/-}$ mice were inoculated. Those mice lack an antiviral response due to the receptor deficiency and, therefore, are highly susceptible to BTV infection (Calvo-Pinilla *et al*. 2009a).

Two mice that were inoculated with blood from alpacas #1 and #2 (samples from 2 to 6 dpi; C_q values 35.4 and 36.3) developed fatal disease and high amounts of BTV RNA were detected in their spleens: 5.6 x 10^7 (C_q 14.0) and 2.9 x 10^8 copies per mg of tissue (C_q 12.4), respectively. The spleens of all other mice were either negative in the RT-qPCR or yielded a C_q value close to the limit of detection.

No virus could be isolated from SAC spleens using IFNAR$^{-/-}$ mice or tissue culture.

3.4. Blood-cell binding experiment

After incubation with BTV-8, significantly more BTV RNA was detected in blood cell pellets (1.7 x 10^7 copies per ml on average) of cattle than in blood cell pellets of SAC (2.0 x 10^6 copies/ml on average). Furthermore, amounts of BTV RNA were significantly higher in the plasma fractions of SAC blood (1.5 x 10^7 copies/ml on average) than those in the plasma fractions of cattle blood (9.5 x 10^6 copies/ml on average) (Fig. 23). In the fractions of pig blood, amounts of BTV RNA were very similar to those found in the blood fractions of SAC (data not shown). After 5 washes, BTV RNA in the supernatant was nearly eliminated in all

species. Pig blood cells deteriorated quickly and no samples could be taken after washing. A significantly higher amount of BTV RNA was retained in packed bovine blood cells (1.1 x 10^7 copies/ml on average) than in blood cells from SAC (1.0 x 10^6 copies/ml on average). Similar results were obtained with blood samples that had been washed before incubation with BTV-8 (plasma replaced by PBS).

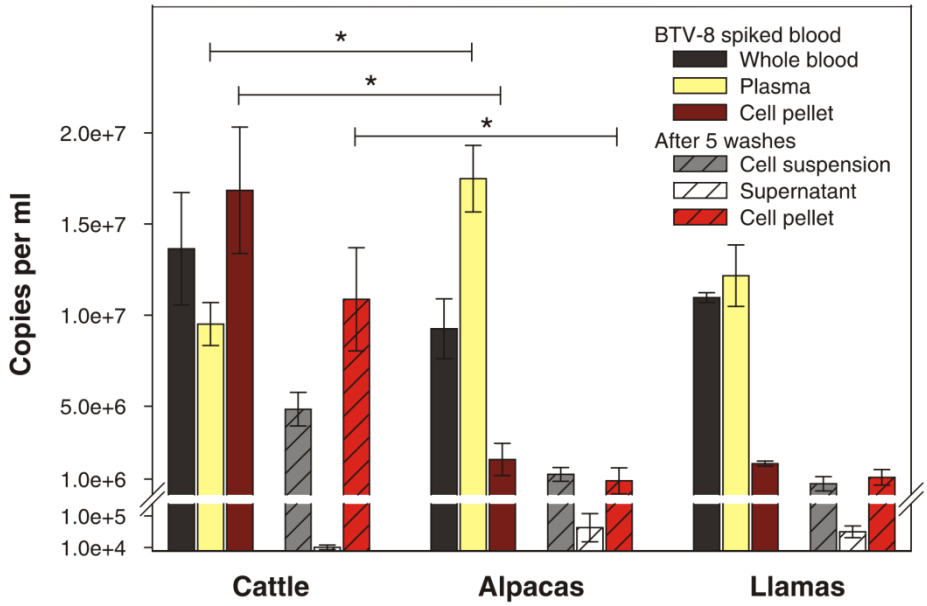


Fig. 23. *In vitro* adsorption experiment of BTV-8: BTV RNA in SAC blood was predominantly found in the plasma fraction. A significantly reduced viral RNA load was detected in the blood cell pellets and was readily eliminated by washing. In contrast, significantly higher amounts of BTV RNA were found in blood cell pellets of cattle than in blood cell pellets of SAC. (*, *p*-value <0.05; error bars indicate standard deviation.)

4. Bluetongue vaccination

This is the first study monitoring the long-term progression of BTV antibodies in llamas and alpacas vaccinated with any one of three inactivated BTV-8 vaccines that were available in Germany at the time. The Bluevac 8 and Zulvac 8 Bovis vaccines have been evaluated for the first time. Furthermore, owners were asked retrospectively by telephone interview whether they had observed any adverse effects p.v.

Neonates obtain passive immunity by ingestion and intestinal absorption of antibodies in colostrum immediately after birth (Weaver *et al*. 2000, Wernery 2001). Transfer of immunoglobulins from the dam to the neonate is a critical factor for protective immunity to a

broad range of diseases (Weaver *et al*. 2000, Vitour *et al*. 2011). However, colostral immunity interferes with the induction of an immune response to vaccination or natural infection and has already been reported in lambs and calves (Oura *et al*. 2010, Vitour *et al*. 2011). Therefore, the transfer and progression of colostrum-derived BTV-8 antibodies in SAC crias born from vaccinated dams was monitored.

All serum samples were tested with a DR ELISA, and sera from dams and their crias born after vaccination of their mothers were analysed for neutralising antibodies using SNT. Both tests were validated by the animal experiment (see respective section).

4.1. Vaccination study

In this field study, we monitored the long-term progression of BTV antibodies in 65 SAC from 5 different herds that were vaccinated with an inactivated BTV-8 vaccine. Since SAC species and the used vaccine and dosage differed in each herd, the results of the 5 herds were evaluated individually (Table 12). The seroconversion rates given are all based on the results of the DR ELISA.

The six negative controls as well as the alpacas that had not seroconverted after vaccination remained seronegative during the whole study. In SAC vaccinated twice, the seroconversion rate was 93% (43 of 46) 3 to 4 weeks p.v., and 100% after 5 to 6 months. Herd 3, which received a single vaccination, showed a seroconversion rate of 79% (15 of 19) after 6 months (Table 12 and Fig. 24). In 2009, seven alpaca crias were born in herd 2 with colostral BTV antibodies that disappeared 5 to 9 months p.n. (see also IV.4.3). As soon as these "sentinel" crias became negative, they were tested repeatedly negative until the end of the study (Fig. 25).

Table 12. Seroconversion rates in 65 SAC after BTV-8 vaccination as measured by double recognition (DR) ELISA.

Herd #	Species	No. of SAC	Vaccine label	Dosage (s.c.)	Seropositive after 3-4w p.v.		Seropositive 5-6m up to 1[b] or 1.5y[c] p.v.	
					No.	%	No.	%
1	Alpaca	14	C	2 x 2 ml	13	93	14	100[b]
2	Alpaca	15	C	2 x 2 ml	15	100	15	100[c]
3	Alpaca	19	C	1 x 2 ml	ND	ND	15	79[b]
4	Llama	9	M	2 x 1 ml	7	78	9	100[c]
5	Llama	8	F	2 x 2 ml	8	100	8	100[b]

[a] Lot numbers available upon request; s.c., subcutaneous; p.v., post vaccination; w, weeks; m; months; y, years; ND, not determined.

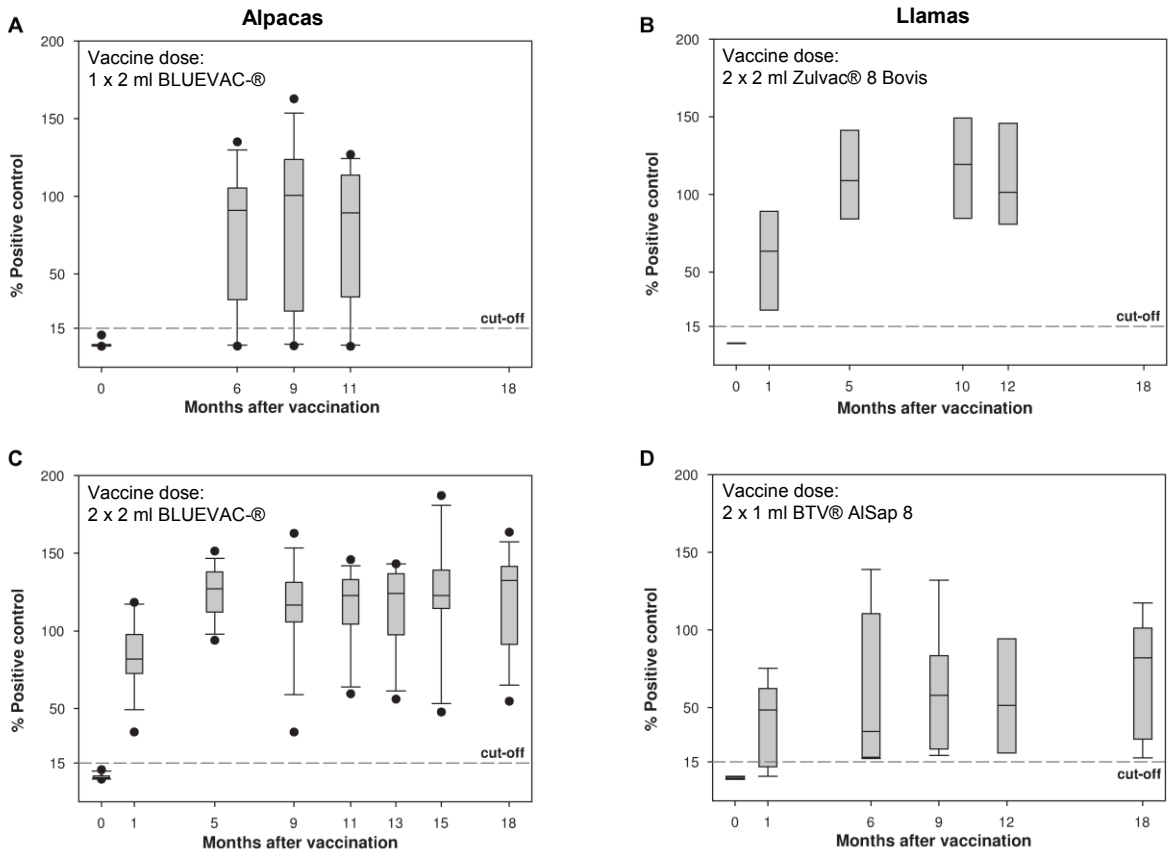


Fig. 24. Development of BTV antibodies in BTV-8 vaccinated SAC through one year (A: herd 3, B: herd 5) or 1.5 years (C: herd 2, D: herd 4) after vaccination as measured by DR ELISA. Seroconversion rates and the variance of antibody levels depended on the dose of vaccine given. This chart includes data from vaccinated crias, but not from crias with maternally derived antibodies. The boxes indicate the lower and upper quartiles and the median. The ends of the whiskers mark the 10th and 90th percentiles. Individual outliers outside of that range were plotted as dots. Whiskers and dots were omitted if there were not enough data points.

In herds that were also tested by cELISA, the detected seroconversion rates were significantly lower in the cELISA (39%, 9 of 23) than in the DR ELISA (80%, 20 of 23). According to Fleiss' criteria (Fleiss 1981), the point estimate of kappa (0.18) suggests poor agreement between the two ELISA systems. However, all animals positive in the cELISA were also positive in the DR ELISA. All pre-immunisation sera were negative in both assays.

In SAC vaccinated twice with 2 ml of vaccine, the variance of antibody levels 5 to 6 months p.v. was lower than in SAC vaccinated with a single dose or twice with 1 ml. While this difference was significant, no significant difference in the variance of antibody levels was found in later samples taken 9 to 12 months p.v. (Fig. 24). Peak antibody levels were reached in all herds within 5 to 10 months after vaccination and remained stable until the end of the

study (11 to 18 months after vaccination) (Fig. 24). Eight seropositive SAC could not be followed until the end of the study because they had been sold or had died of causes unrelated to BTV infection.

4.2. Tolerance of BTV-8 vaccines

Apart from the animals that participated in the vaccination study, vaccinations in additional SAC were documented retrospectively by telephone questionnaire. No adverse effects were reported in 97.7% (339 of 347) SAC on 27 farms (including animals of the vaccination study), which were vaccinated with any one of four inactivated BTV-8 vaccines. The maximum vaccine dose used for alpacas was 2 x 2 ml of vaccine C or twice 1 ml of vaccine M. In llamas, the maximum dose was 2 x 4 ml of vaccine C, 2 x 2 ml of vaccine F, or two 1 ml doses of Bovilis® BTV8 (Intervet, Boxmeer, The Netherlands). Booster vaccinations in two consecutive years were well tolerated by 46 alpacas and 29 llamas.

No irregularities concerning pregnancies, birth rates or health status of over 95 crias born from 2008 to 2010 were observed by the owners of herds 1 to 5.

For the herds that did not participate in this vaccination study, one owner reported local swelling at the injection site in 5 alpacas. On two other farms, two fatalities after vaccination occurred in one 3-year-old alpaca male and one 1.3-year-old alpaca female. Both animals were vaccinated once with 2 ml of vaccine C. The 3-year-old alpaca male died suddenly during the night following vaccination. According to the post-mortem report, a causal link to vaccination has not been identified. The 1.3-year-old alpaca female perished four weeks p.v. A 1-year-old alpaca male on the same farm showed a reduced general condition and buccal ulcerations but recovered after 6 weeks. On the latter two farms, other alpacas (33 overall) were vaccinated but did not show any adverse effects.

4.3. Maternal BTV antibodies in crias

Maternally-derived BTV antibodies were found in all crias from vaccinated dams except in one cria (10) that was not tested before 19 weeks of age – at a time when about half of the other crias were already negative in the ELISA (Fig. 25).

Positive results in the DR ELISA were obtained from 1 week p.n. (cria 1) for at least 7 weeks (cria 2) up to a maximum of 30 weeks (cria 6) (median age at last positive sample: 14 weeks). Of all initially seropositive crias born in 2009, 5 of 8 (63%) became seronegative between week 20 and 27 p.n. (crias 1, 2, 4, 5, 7), and 3 (27%) between week 30 and 39 (crias 3, 6, 9) (median age: 26 weeks) (Fig. 25).

NAbs were only detected in llama cria 9 (six weeks p.n.), which was born 3 months after the mother had been vaccinated with vaccine F. Compared to the other dams, this dam displayed the highest NAb titre 3 weeks p.v. and around parturition (Table 13). All other crias born over 5 months p.v. of their mothers were found negative irrespective of the NAb status of the dam. Three weeks after the 2nd vaccination, 7 of 9 dams had NAb, while only four were positive around parturition 3 to 11 months p.v. No correlation was found between NAb titres and antibody levels measured by DR ELISA (Table 13).

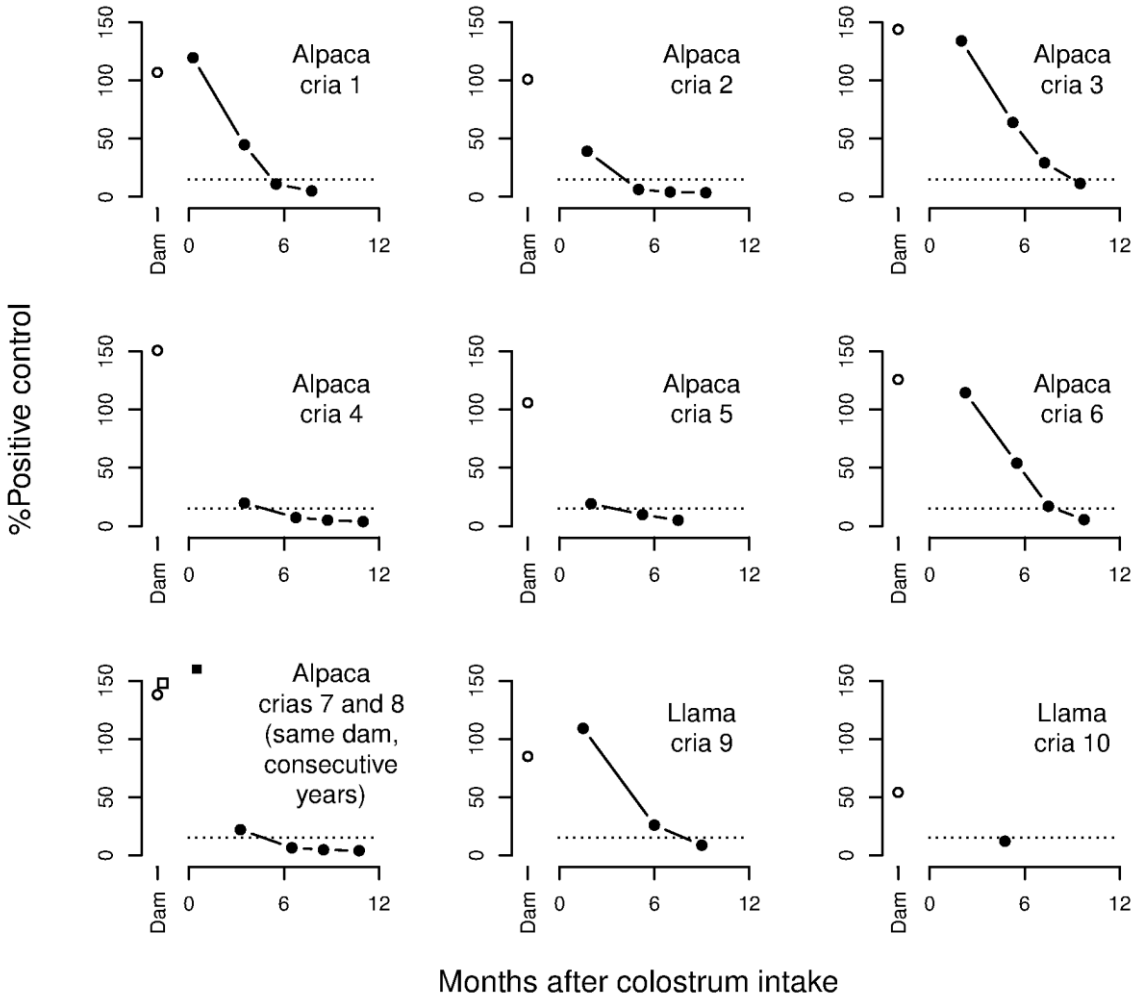


Fig. 25. Transfer of colostral BTV-8 antibodies to crias. Antibody levels of BTV-8 vaccinated dams at the time of parturition ("Dam"), and their crias after birth as measured by DR ELISA. The progression of colostrum-derived BTV antibody levels was similar in all crias with a high initial level. Blood samples from the dam of crias 7 and 8 were taken in 2009 and 2010, respectively. While antibody levels of dam 7 remained stable within the year, the antibody levels in her crias diverged considerably (2009: ○ dams, ● crias; 2010: □ dam 7, ■ cria 8).

Table 13. BTV-8 antibody levels in dams and their crias measured by serum neutralisation test (SNT) and DR ELISA.

Herd	Dam						Cria			
#, V	#	3 weeks p.v.		Around parturition			#	After birth (p.n.)		
		SNT ND_{50}	ELISA % PC	SNT ND_{50}	ELISA % PC	Months p.v.		SNT ND_{50}	ELISA % PC	Weeks p.n.
2, C	1	4	82	Neg	107	8	1	Neg	120	1
2, C	2	4	35	Neg	101	7	2	Neg	39	7
2, C	3	Neg	73	Neg	144	6	3	Neg	134	8
2, C	4	6	118	3	151	5	4	Neg	20	14
2, C	5	Neg	59	Neg	106	9	5	Neg	19	12
2, C	6	12	76	3	126	6	6	Neg	115	9
2, C	7	3	97	Neg	138	5	7	Neg	22	13
				Neg	148	18	8	Neg	160	2
5, F	9	16	36	12	85	3	9	8	109	6
5, M	10	4[a]	31[a]	4	54	11	10	Neg	Neg	19

#, herd or animal number; V, vaccine; p.v., post vaccination (after the 2nd vaccination); p.n., post natum; ND_{50}, neutralising dose: reciprocal of the serum dilution that caused virus neutralisation in 50% of the replicates; % PC, percent positive control: % PC ≤ 15 is negative, % PC > 15 is positive; Neg, negative; [a] the sample was collected eight months p.v. as none was available 3 weeks p.v.

VI. DISCUSSION

1. Entomological monitoring

1.1. Influences on ceratopogonid abundance and BTV infection rates in SAC herds

Knowledge of the distribution, habitats and factors influencing the abundance of vectors of emerging diseases is crucial to predict a possible introduction and spread of emerging diseases, and to implement appropriate precautionary or control measures against outbreaks of vectorborne diseases (Purse *et al*. 2005, Kampen and Kiel 2006). Species belonging to the Obsoletus and Pulicaris complexes have been incriminated as the main potential BTV vectors in northern Europe, and *C. dewulfi* in the Netherlands (Meiswinkel *et al*. 2008). The

proportions of *C. obsoletus s.l.* and *C. pulicaris s.l.* were similarly high compared to those reported by other groups in northeastern Europe (Meiswinkel *et al*. 2008, Bartsch *et al*. 2009, Hörbrand and Geier 2009, Vorsprach *et al*. 2009, Mehlhorn *et al*. 2009a, Mehlhorn *et al*. 2009c). However, *C. pulicaris s.l.* never exceeded the proportion of *C. obsoletus s.l.* in the collections, which was occasionally observed at other locations in Germany in May/spring (Kiel *et al*. 2009, Clausen *et al*. 2009, Bartsch *et al*. 2009, Hoffmann *et al*. 2009a, Mehlhorn *et al*. 2009b) and frequently around outbreak sites in Belgium (de Deken *et al*. 2008). In the present study, *Culicoides* abundance and BTV seroprevalence in herd A were considerably higher than in herd B, although both herds were located in regions where the risk for BTV-8 infection was high at the time (Conraths *et al*. 2009) (Fig. 18) (Schulz *et al*. 2012c). Statistical analysis revealed a strong effect of the location variable on the number of *Culicoides* on the farms. Anthropogenic, landscape, meteorological, biotic and abiotic factors can have an influence on vector abundance and prevalence of BTV infection in ruminants (Conte *et al*. 2007, Hörbrand and Geier 2009, Vorsprach *et al*. 2009, De Liberato *et al*. 2010, Purse *et al*. 2012, Mayo *et al*. 2012a). Seasonal and meteorological effects (especially temperature, humidity and wind speed) belong to the key factors influencing the abundance and diversity of *Culicoides* (Mellor *et al*. 2000, Mellor *et al*. 2009b, Purse *et al*. 2012). Similar to results of other groups (Mellor *et al*. 2000, Kiel *et al*. 2009, Vorsprach *et al*. 2009), the interaction of temperature and humidity also had a significant effect on the number of *Culicoides* in the present study. Hence, the association with the listed factors can explain the annual deviations in the abundance and composition of *Culicoides* and other ceratopogonid genera observed on farms B and C. *Culicoides* abundance peaked in July and August 2008 in the present study. However, the highest incidence of vectors and of BT disease in temperate areas is usually recorded in late summer and autumn (Hoffmann *et al*. 2009a, Mellor *et al*. 2009b). Cool weather conditions in late summer might have influenced the earlier decrease in the number of *Culicoides* (Hörbrand and Geier 2009). On the other hand, results were contradictory analysing the three SAC farms separately, indicating that additional factors were not included in the regression model that might have influenced the results. Factors that possibly had a positive effect on the number of *Culicoides* on farm A and that differed from local conditions on farms B and C (Table 2) included a high land cover with natural-wood, location at about 300 m a.s.l. and in a valley (with light winds) (Bartsch *et al*. 2009, Mehlhorn *et al*. 2009c, De Liberato *et al*. 2010).

Besides, the high number of cattle kept near SAC herds could have negatively influenced the number of *Culicoides* and seroprevalence in SAC on farms B and C. Cattle was found to

have a 'dilution effect' on the risk of BTV infection (Durand *et al*. 2010a) and to be the most attractive host for Palearctic biting midges (Bartsch *et al*. 2009). On the other hand, the daily maximum of *Culicoides* caught in one trap on farm A was similar to the maximum reported on a dairy farm in central Germany (Clausen *et al*. 2009). De Liberato *et al.* (2010) suggested that the number of farmed animals does not affect *C. obsoletus* densities and/or that the vectors use alternative hosts, but that the abundance of *C. obsoletus* probably rather depends on the availability of shady vegetation. Hence, other factors than the animal species probably had a stronger influence on *Culicoides* abundance and, therefore, BTV infection rates.

The absence of *Culicoides* in winter found in the present study can be explained by the colder winter 2008/2009 compared to the winters in the two previous years (www.dwd.de). According to recent studies, a vector-free period in winter does not exist in Germany and western adjacent countries (Mehlhorn 2009, Meiswinkel 2008, Hoffmann 2009, Clausen 2009), but is possible at certain locations (Clausen *et al*. 2009). A lack of sensitivity of the monitoring systems (Meiswinkel *et al*. 2008) can be precluded since we used the same traps as in the previous monitoring programmes in Germany (Hoffmann *et al*. 2009a).

In the present study, *C. obsoletus* was found to be the only species that occurred during the whole study period (except during the winter months), while *C. pulicaris* was less frequently collected on farms B and C. *C. obsoletus* was named as the most robust *Culicoides* spp. as it can occur in Germany throughout the year (Kiel *et al*. 2009, Vorsprach *et al*. 2009). In contrast, the phase of activity of *C. pulicaris s.l.* is shorter during the year (Mehlhorn *et al*. 2009c).

Only a few individuals of the taxon *C. dewulfi* were collected on farm A. This species exclusively breeds in cattle or horse dung (Meiswinkel *et al*. 2008). However, no cattle were kept on or nearby farm A. To the best of our knowledge, no studies of *Culicoides* instars in SAC dung have been conducted. Therefore, this species possibly also breeds in other habitats, such as dung of SAC, or it has been transported to the valley by winds (Mellor *et al*. 2000). *C. imicola*, the main Afro-Asian vector of BTV (Meiswinkel *et al*. 2004), has so far never been detected north of the Alps (Hoffmann *et al*. 2009a), and it was nor collected on the SAC farms in this study.

The high number of *C. obsoletus s.l.* and *C. pulicaris s.l.* collected in indoor traps indicated that the risk for BTV infection is high in stables, which was already reported elsewhere (Clausen *et al*. 2009). Although the number of ceratopogonid genera, *Culicoides* spp. and proportion of males and females in indoor and outdoor collections depended on the farm, *C. obsoletus s.l.* were generally predominantly collected indoor, while a higher number of *C.*

pulicaris s.l. were caught outdoor. This is in accordance with the strong endophilic and exophilic behaviour of *C. obsoletus/C. scoticus* and *C. pulicaris s.s.*, respectively, reported in Europe (Meiswinkel *et al*. 2008).

The abundance and species composition of *Culicoides* collected for this study was probably biased due to the fact that the traps were only run from dusk until dawn, and a significant number of midges might have gone undetected because they are biting during the day (Meiswinkel *et al*. 2008, Balenghien *et al*. 2008, Clausen *et al*. 2009). On the other hand, many *Culicoides* are active from dusk until dawn as nocturnal activity may reduce the risk of desiccation (lower temperatures, higher humidity) (Mellor *et al*. 2000).

1.2. Phenology of ceratopogonids

The diversity of species found on the three study farms corresponded to the number of species reported by the groups that monitored *Culicoides* in nearby regions in the previous year (Kiel *et al*. 2009, Vorsprach *et al*. 2009, Hörbrand and Geier 2009). Similar to those studies, peaks in the diversity of species were found between June and October, and the number of species caught by months depended on the location (Kiel *et al*. 2009, Vorsprach *et al*. 2009) (Table 5) and year.

1.3. Absence of BTV RNA

None of the analysed *Culicoides* were found positive for BTV RNA, although most of the tested midges were collected on the farm with the highest seroprevalence, and in the same months when the highest number of BTV infected *Culicoides* (*obsoletus s.l.*) and BT cases in ruminants were recorded in Germany in the previous two years (Hoffmann *et al*. 2009a, Mehlhorn *et al*. 2009a). Furthermore, the BTV-8 epizootic was still ongoing at the time of the study (Conraths *et al*. 2009). However, positive batches were only found on farms where the proportion of BTV-infected ruminants was very high (Hoffmann, unpublished observations). On the other hand, the infection of the SAC might have already occurred earlier in the year or during the peak of the epizootic in 2007.

Differences in the pathogenesis of BTV-8 in SAC compared to ruminants could also explain the negative virological results. In contrast to the long-term viraemia characteristic for BTV infection in ruminants, only low amounts of BTV RNA were detected for a short time in SAC experimentally infected with BTV-8 (maximum 5 weeks) (Fig. 22) (Schulz *et al*. 2012b). In the cross-sectional study (Schulz *et al*. 2012c), none of 249 seropositive SAC (including herds

A-C) were BTV RNA positive at the time of the present study. Therefore, the transmission cycle between SAC and *Culicoides* was possibly not maintained in the herds.

1.4. Breeding habitats and occurrence of males

For targeted vector control, it is important to know the *Culicoides* breeding habitats (Werner *et al*. 2012) and where swarming and mating takes place. *Culicoides* males do not feed on blood but on plant juice. Thus, they are not directly important in the transmission cycle of BTV. Peaks in the proportion of Obsoletus complex males caught in black-light traps were also found in April and November by Vorsprach *et al.* (2009). Males typically emerge a short time before females (Mullen 2009), which was also found for *C. obsoletus* males caught in emergence traps in the present study (Fig. 17). In black-light traps, a proportion of generally 0 to 5% of *Culicoides* males (Mehlhorn *et al*. 2007, Bartsch *et al*. 2009, Vorsprach *et al*. 2009, Kiel *et al*. 2009) and an unbalanced monthly occurrence of *C. pulicaris s.l.* males were also recorded by other authors (Kiel *et al*. 2009). Possible reasons for the huge differences in gender distribution can be that black light predominantly attracts females and that the mating and swarming places are not close to the feeding places (hosts) (Mehlhorn *et al*. 2007, Vorsprach *et al*. 2009). In contrast, those places might be close to the breeding habitats, which would explain the considerably higher proportion of males collected in the emergence traps. Furthermore, males usually travel much shorter distances than females (Mullen 2009).

We have no suitable explanation for the considerably lower proportion of *Culicoides* males (14.1% of 185) and Obsoletus complex males (14.3%) collected in the faeces trap than in the pasture trap (68.2% respectively 66.7%). A distortion of data by female midges escaping from traps in search of a host or slipping in from outside to lay their eggs should be taken into consideration. The markedly higher number of *Culicoides* caught in the faeces trap indicates that adult *Culicoides* prefer faeces (or the subjacent ground) for breeding and their larvae as feeding substrate, which was already described by Bishop *et al*. (1996) and Boorman *et al*. (1986). On the other hand, substrate preferences depend on the *Culicoides* spp. (Werner *et al*. 2012). In the present study, *C. albicans* were predominantly caught in the pasture trap, while *C. obsoletus s.l.* and *C. achrayi* presented the dominant taxa in the faeces trap. Similar to results of a previous study (Werner *et al*. 2012), *Culicoides* were most frequently collected in late summer, but in this study, a peak was also recorded in May (Fig. 17). Those peaks probably indicate that hatching of *Culicoides* (especially *C. obsoletus s.l.* and *C. achrayi*) predominantly takes place during these months. The most frequent species caught in the

faeces trap belonged to the Obsoletus complex, which is in accordance with results reported by Werner *et al*. (2012).

2. Cross-sectional study

The epidemiological background of BTV infection, associated risk factors and its clinical expression have so far only been investigated in ruminants, but never in any detail in SAC. The fatalities in a few SAC that occurred during the recent BTV-8 and BTV-1 epizootics in Germany and France, respectively, prompted the investigation of their role in the epidemiology of BTV.

A total of 14.3% of tested SAC were positive for BTV antibodies in the cross-sectional study in Germany from autumn 2008 to spring 2009. The spatial distribution of BTV-8 infection in SAC was similar to the BTV-8 outbreaks registered in ruminants in Germany between 2006 and 2008 (Conraths *et al*. 2009). However, BTV-8 infection in domestic ruminants in Germany had not been thoroughly investigated. Therefore, a direct comparison with our data is not possible. The estimated proportion of BTV infection in exposed domestic ruminants in Germany in 2007 was considerably lower (below 6.4%) (Conraths *et al*. 2009) than in exposed SAC (in seropositive herds) in the present study (27.6%). In contrast, markedly higher seroprevalences were reported in domestic ruminants (cattle, sheep, goat) and red deer in northwestern Europe (34–70%) (van Schaik *et al*. 2008, Lutz 2008, Elbers *et al*. 2008c, Linden *et al*. 2010) where the BTV-8 epizootic had initially started in 2006 (Conraths *et al*. 2009). This is consistent with the BTV-8 seroprevalence found in SAC in the west of Germany (42.7%, Table 8 and Table 1 in Appendix 4). BTV antibodies and BTV-1 RNA have been detected in a few llamas during an outbreak in France (Meyer *et al*. 2009), but no BTV antibodies have been detected in 354 SAC in Switzerland. This can be explained by the higher pressure of BTV infection on SAC populations in France and Germany as more BTV outbreaks occurred in those countries than in Switzerland (Zanolari *et al*. 2010b).

BTV-8 seroprevalence in infected SAC herds varied widely (2–100%) as it was also described in ruminant herds (cattle, sheep, goats) in the Netherlands (van Schaik *et al*. 2008, Elbers *et al*. 2008c). Variations in BTV infection rates occur regularly in different ruminant species and SAC (Rivera *et al*. 1987, Mattson 1994, Conraths *et al*. 2009, Meyer *et al*. 2009, Falconi *et al*. 2011). Seroprevalence in SAC herds generally decreased with distance from the region where the first BTV-8 outbreaks were recorded, and location was significantly associated with BTV infection. The location variable can be considered a proxy for BTV exposure in the respective regions. Geographical variation in BTV seroprevalence was reported by several groups (Ruiz-

Fons *et al*. 2008, Elbers *et al*. 2008c, Durand *et al*. 2010b, García-Bocanegra *et al*. 2011). Different ecosystems and vector/pathogen distribution were suggested to have a substantial influence on BTV diffusion and exposure rate, respectively, while the amplification of BTV circulation in affected herds played a rather limited role (Ruiz-Fons *et al*. 2008, Durand *et al*. 2010b). Nevertheless, the pressure of infection on naïve animals in areas closer to those regions where BTV has occurred first will be higher than in regions that are farther away from those hot spots.

As of 2011, the population of SAC in Germany is estimated at about 7,000 animals (Locher *et al*. 2010, Schulz *et al.*, manuscript in preparation). Most (94%) of the contacted owners that met the requirements participated in this study, and the sample reflects the SAC population recorded in 227 herds in Germany (Schulz *et al.*, manuscript in preparation). Therefore, the random sample is considered representative. The overall sample size was overfulfilled, but for samples taken within herds, sample sizes in almost 40% of the herds were smaller than required to detect at least one seropositive animal in the herd (Table 3). This might have resulted in an underestimation of the number of positive herds. On the other hand, (i) the overall seroprevalence in Germany was higher than 5%, (ii) the within-herd seroprevalence varied considerably (2–100%; Table 8), and (iii) seroprevalence was generally lower in the eastern than in the western half of Germany (Fig. 18, Table 8 and Table 1 in Appendix 4). As described earlier, other factors apparently had an influence on seroprevalence in a herd. Furthermore, the table used to determine the sample size on herd level is designed for contagious and not for vectorborne diseases, which have a different distribution pattern (Wilson *et al*. 2007). Thus, it is particularly questionable whether sample sizes were generally sufficient at the edges of regions that had been affected by the BTV-8 epizootic wave and where within-herd seroprevalences were probably lower than the assumed 5%. Therefore, it seems likely that the number of positive herds was generally underestimated in eastern regions.

A possible distortion of our data due to animal movement was identified for five seropositive animals in one herd that had been shipped to Bavaria in 2008. Missing information due to an interview bias cannot be excluded, but a relevant distortion of data is unlikely as the spatial distribution of BTV outbreaks in ruminants in the same period was very similar (Conraths *et al*. 2009). The used ELISA does not discriminate between serotypes, and a positive result due to BTV infection with another serotype cannot be ruled out. During the time of the study, BTV-6 had been detected in cattle in a field survey in northwestern Germany in late 2008 (Eschbaumer *et al*. 2010a). Only 16 blood samples of SAC from that area were seropositive

(Table 1 in Appendix 4), and no BTV RNA was found in those samples, which would have indicated a recent infection with any other BTV serotype at the time. Furthermore, the proportion of cattle positive for BTV-6 antibodies was very low (Eschbaumer *et al*. 2010a). Therefore, a measurement bias for the overall proportion of BTV-8 antibodies detected in SAC is considered negligible.

Location and age were the only risk factors for BTV infection that remained significant in the logistic regression model (Table 10). Analysing interaction terms, only the location remained a significant factor. This means that the univariate results were confounded by the location variable because the majority of seropositive herds were located in the western half of Germany where the risk of BTV exposure was significantly higher than in the eastern half (Fig. 18, Table 8). The effect of age on BTV infection was not significant in the analysis of interaction terms. However, we assume that there still is an association with the outcome variable because the OR and 95% CI were almost identical in the logistic regression and the univariate analyses (data not shown). A significant association between age and BTV seroprevalence was reported by several groups (Ward *et al*. 1994, Linden *et al*. 2010, García-Bocanegra *et al*. 2011), and can be explained by a longer time of exposure for adults (García-Bocanegra *et al*. 2011, reviewed by Falconi *et al*. 2011). Interestingly, a high proportion of seropositive dams (35 of 55) also had seropositive crias (data not shown), and we assume that their crias have passively obtained immunity by the colostral transfer of BTV-8 antibodies. Colostral transfer of BTV antibodies to neonates was already recorded by other authors, and care must be taken when interpreting the presence of antibodies in serum samples from juveniles (Falconi *et al*. 2011) (see VI.4.3). The study design generally included non-weaned crias from tested dams, which has probably caused a distortion towards a higher proportion of seropositive crias and a higher overall seroprevalence.

Species, sex, housing type and presence of other ruminants are risk factors that can influence BTV infection rates in ruminants (Ward *et al*. 1994, Ruiz-Fons *et al*. 2008, Santman-Berends *et al*. 2010, Durand *et al*. 2010b, Falconi *et al*. 2011). However, in SAC no significant association with BTV infection was found for any risk factors other than location and age. The risk of BTV infection in a SAC population is obviously due to BTV exposure, pressure of infection and age rather than other risk factors.

The absence of BTV RNA in the field samples of SAC despite the high seroprevalence and the ongoing BTV-8 epizootic can be explained by the collection of samples late in the season, after vectorborne exposure had mostly abated (Hoffmann *et al*. 2009a), and by the lower pressure of BTV-8 infection in 2008 compared to 2007 (Conraths *et al*. 2009). Furthermore,

BTV RNA in SAC blood can only be detected for a considerably shorter time after BTV-8 infection (up to 5 weeks) than in ruminant blood as BTV-8 adsorbs less efficiently to SAC erythrocytes than to bovine RBC (Fig. 23) (Schulz *et al*. 2012b). Seronegative samples were not analysed for BTV RNA, and theoretically some BTV RNA-positive animals might have been missed. However, the underestimation of BTV RNA prevalence is considered low because in SAC the time period between BTV infection and seroconversion is only about one week (Afshar *et al*. 1995a, Schulz *et al*. 2012b) (Table 11), and most samples (91%) were taken outside the main vector season (Hoffmann *et al*. 2009a).

Severe disease was reported in domestic ruminants – particularly sheep – during the BTV-8 epizootic (Dal Pozzo *et al*. 2009b). In wild ruminants, BTV infection can also result in severe clinical signs, but this depends on the susceptiblity of the affected species. For example, no clinical signs were recorded in red deer experimentally infected with European BTV-8 (López-Olvera *et al*. 2010, Falconi *et al*. 2011). In the present study, clinical signs of BT were not observed by the owners in any of the tested SAC. This is consistent with earlier studies by Rivera *et al*. (1987) and Mattson *et al*. (1994). If there was BT disease in the herds in the present study, the owners might have failed to recognise it because the clinical signs were unspecific and mild (Schulz *et al*. 2012b). Differences in the manifestation of BT disease in various ruminant species and Old World camelids were also reported in other studies (Conraths *et al*. 2009, MacLachlan *et al*. 2009, Batten *et al*. 2011). On the other hand, in the three recorded BT cases presented here, BTV RNA was detected at post-mortem examination, and BT disease was assumed in one perished llama. BTV-related disease and consequent fatalities in alpacas and llamas were recently reported during the BTV-8 and BTV-1 epizootics in Germany and France, respectively (Henrich *et al*. 2007, Meyer *et al*. 2009), and in the USA (Ortega *et al*. 2010b). However, whether BTV infection in the three fatal cases exacerbated concurrent diseases or vice versa, or whether the detection of BTV RNA was only a secondary pathological finding remains unclear. Immunosuppression can aggravate BTV infection in ruminants (Brodie *et al*. 1998). All three cases ocurred in winter when infectious midges are unlikely to be found (Wilson *et al*. 2007, Hoffmann *et al*. 2009a).

3. Animal experiment

The range of hosts susceptible to BTV infection includes domestic and wild ruminants as well as camelids. In SAC, serological evidence for a previous BTV infection was detected in Peru (20%, n=114, Rivera *et al*. 1987), Oregon, USA (1.5%, n=270, Mattson 1994), France (78%, n=9, Meyer *et al*. 2009) and in Germany 2008/2009 (14.3%, n=1742) (Schulz *et al*. 2012c).

However viraemia, clinical signs, abortion and fatalities related to BTV infection have been reported only rarely (Fowler 1998c, Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b).

Experimental BTV infection of camelids was conducted in two llamas – to evaluate antibody response to BTV infection in a cELISA – (Afshar *et al*. 1995a) and recently in three dromedary camels (Batten *et al*. 2011). Based on the results, camels were suggested to have a potential role in the transmission of BTV-1 to local *Culicoides* vectors in Morocco, and they were named as possible reservoirs to carry the virus over long distances (Batten *et al*. 2011). However, to date no experimental infections had been conducted to evaluate viraemia and infection dynamics in SAC after BTV infection.

Mild unspecific clinical signs such as anorexia, arched backs or lung sounds, which were found in two llamas, as well as extended recumbency, mild conjunctivitis and leukopenia, which were found in all SAC between 2 to 8 dpi, were also recorded for ruminants after BTV infection (Ellis *et al*. 1990, Foster *et al*. 1991, Darpel *et al*. 2007, Darpel *et al*. 2009b) (reviewed by MacLachlan *et al*. 2009, Dal Pozzo *et al*. 2009a). Reports of fatal BTV infection in SAC have described a short period of lethargy or weakness, recumbency, respiratory distress and anorexia (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b). However, the severity of BT in the field can be influenced by secondary bacterial infection (Verwoerd and Erasmus 2004, Darpel *et al*. 2007), immune suppression (Brodie *et al*. 1998), or external factors such as stress or sunlight. Therefore, high-containment animal housing might have reduced the displayed clinical signs of BT in this experiment (Verwoerd and Erasmus 2004). On the other hand, no clinical signs were observed in seropositive SAC in Peru (Rivera *et al*. 1987) and Oregon, USA (Mattson 1994).

In the presented animal experiment, the white blood cell count was considerably reduced as long as detectable BTV RNA increased in blood, but had returned to normal values by 20 dpi (Fig. 19). Leukopenia during the first days after BTV infection was also recorded in domestic (Ellis *et al*. 1990, Foster *et al*. 1991, Verwoerd and Erasmus 2004, Martinelle *et al*. 2011) and wild ruminants (Vosdingh *et al*. 1968, Howerth *et al*. 1988). It was suggested that a BTV-specific lymphocyte destruction or lymphocyte sequestering at virus replication sites (e.g. lymph nodes) might be responsible for the lymphopenia (reviewed by Darpel *et al*. 2009b). Whether the leukopenia detected here resulted from a similar mechanism is unknown.

All SAC seroconverted, indicating that they had mounted an immune response to BTV-8 infection. In the DR ELISA, BTV antibodies were detected earlier than in the cELISA and the

SNT (Table 11). Due to the small number of animals in the experiment, this difference was not statistically significant. A similar trend, however, has been reported for ruminants (Eschbaumer *et al*. 2009, Oura *et al*. 2009). In general, seroconversion within two weeks after BTV infection is in agreement with data recorded for experimentally infected domestic and wild ruminants (Foster *et al*. 1991, Darpel *et al*. 2007, Eschbaumer *et al*. 2009, Dal Pozzo *et al*. 2009a, López-Olvera *et al*. 2010, Eschbaumer *et al*. 2010b), camels (Batten *et al*. 2011) and llamas (Afshar *et al*. 1995a). However, in the cELISA, peak antibody levels were reached relatively late (35 to 48 dpi) compared to experimentally infected sheep (10 dpi), cattle (18 dpi) (Darpel *et al*. 2007) and red deer (17 to 28 dpi) (López-Olvera *et al*. 2010), but at a similar time as in camels (after 41 dpi) (Batten *et al*. 2011). About one month after BTV-8 infection, mean neutralising antibody titres in SAC (35 dpi: 7.2 $\log_2$ ND_{50}; Fig. 20 and Fig. 22) were similar to those found in sheep (Eschbaumer *et al*. 2009, 2010b) and cattle (Martinelle *et al*. 2011). All animals remained positive in all assays until the end of the experiment (106 dpi) (Table 11, Fig. 20 to Fig. 22).

Interestingly, a significant decrease of antibody levels almost to the detection limit was observed in the DR ELISA between 20 to 35 dpi (Fig. 21). A similar decline has been observed in experimentally infected sheep and cattle (Eschbaumer *et al*. 2011). DR ELISAs can detect the antibody response to a BTV infection earlier than competitive tests, and are highly sensitive for vaccine-induced antibodies (Eschbaumer *et al*. 2009, Oura *et al*. 2009). Their dependence on immunoglobulin M (IgM) (*Anon*. 2010a), however, might compromise their sensitivity after its peak early in infection. The observed effect is probably related to immunoglobulin class switching. Early in infection, IgM is produced in high amounts, but is soon replaced by other immunoglobulins (Murphy *et al*. 2008). After experimental infection of naïve sheep with *Rift Valley Fever virus*, specific IgM peaked on day 10, rapidly decreased between days 14 and 28 and had almost disappeared by day 42. Specific IgG increased until day 21 and then remained quite stable (Paweska *et al*. 2003).

Both kinds of commercial BTV ELISAs use plates coated with recombinant BTV antigen. In the sandwich (double-antigen) assays, captured multimeric immunoglobulins from the specimen can bind more soluble antigen conjugate and will give a stronger signal than molecules with only two antigen binding sites close together such as IgG. A low sensitivity for IgG could explain the weak signal three to four weeks after infection. The reason for its eventual recovery, however, presently remains unclear and requires further investigation. A possible role of serum IgA has been suspected.

Signal strength in cELISAs, on the other hand, depends on the availability of unblocked BTV antigen on the plate, independent of the class of the blocking antibodies in the sample. Therefore, cELISAs do not react recognisably stronger to IgM than to IgG, even though the larger IgM molecules probably block antigen by steric interference in addition to specific binding.

For BTV diagnosis, DR ELISAs have clear advantages. They can detect an immune response very early after infection, and are highly sensitive for antibodies induced by vaccination with inactivated vaccines. If they are used exclusively, however, there is a risk of false-negative results for animals that were infected and might still be viraemic. For a safe serological diagnosis, it is recommended that samples should be tested with a cELISA first. If the cELISA is negative, a DR ELISA can be used for further analysis.

Using RT-qPCR, BTV RNA was first detected at 2 dpi in every animal, with the lowest C_q values occurring around 8 dpi (Fig. 22). This is consistent with findings in sheep (Foster *et al*. 1991), cattle (Dal Pozzo *et al*. 2009a) and red deer (López-Olvera *et al*. 2010). In experimentally infected camels, BTV-1 RNA was first detected several days later (5 to 8 dpi), and the lowest C_q values were reached inconsistently around 7 to 15 dpi (Batten *et al*. 2011).

Possible explanations for the two-peak dynamics of viral genome load in peripheral blood (Fig.22) are (i) a biphasic viraemia characterised by a first peak due to virus release from local replication sites, and a second peak owing to massive virus production at secondary replication sites (mostly ECs and peripheral blood mononuclear cells) (reviewed by MacLachlan *et al*. 2009); or (ii) a short-time reduction of viral genome corresponding to an interferon-induced reduction of the viral titre as described for sheep (Foster *et al*. 1991).

Interestingly, the amount of BTV RNA detected by RT-qPCR was considerably lower in SAC than in ruminants – even at the peak around 8 dpi. Similarly high C_q values were reported for camels (Batten *et al*. 2011). Seroconversion correlated with the decrease of BTV RNA in SAC blood (Fig. 22), which was finally cleared after 28 to at most 35 dpi. No infectious virus was detected by Vero cell culture or ECE from whole blood, plasma or blood cell pellets at any time point. This includes samples taken before seroconversion. However, using the highly sensitive $IFNAR^{-/-}$ mouse model (Calvo-Pinilla *et al*. 2009a), replication-competent virus could be demonstrated in peripheral blood of two experimentally inoculated alpacas between 2 to 6 dpi; before the first detection of BTV antibodies. In camels, BTV-1 isolation had been successful on KC cells (from *Culicoides sonorensis*) at 7 and 8 dpi (Batten *et al*. 2011).

The findings of reduced viral load in SAC are considerably different from those in ruminants, as one of the main characteristics of BTV infection is a prolonged viraemia with co-circulation of virus and neutralising antibodies. Infectious virus could be reisolated from the blood of ruminants for up to three months, and BTV RNA was detectable for up to 7 months after infection (Brewer and MacLachlan 1992, Bonneau *et al*. 2002).

Spleens collected at post-mortem examination were positive for BTV RNA using RT-qPCR, although SAC blood had already turned negative 71 to 78 days before. Similar results have been reported by Worwa *et al*. (2010). Spleens of sheep euthanised 151 dpi were positive in a RT-qPCR. However, those necropsies were conducted only 11 to 25 days after the last detection of BTV RNA in blood. No infectious virus could be isolated from the SAC spleens in Vero cells or highly susceptible IFNAR$^{-/-}$ mice. This was also found for the sheep spleens by Worwa *et al*. (2010) using KC and Vero cells.

The seeming persistence of BTV RNA in sheep spleens has been explained as an accumulation of viral remnants due to the clearance of senescent virus-carrying erythrocytes (Worwa *et al*. 2010). SAC spleens, however, were positive for BTV RNA with similar C_q values as sheep spleens, even though BTV does not efficiently bind to SAC blood cells (Fig. 23). Therefore, erythrocyte clearance probably cannot explain the prolonged presence of viral RNA in the spleen in this case. More likely, it is due to the association of BTV with EC, macrophages and lymphocytes as demonstrated in ruminant spleens by immunolabelling (Darpel *et al*. 2009b, Sánchez-Cordón *et al*. 2010).

BTV RNA levels detected in whole blood samples of SAC were similar to those detected in the serum fraction. Furthermore, a very low amount of BTV RNA or none at all was detected in the blood cell fraction. This contrasts to results from infected ruminants where BTV RNA is generally found in considerably lower amounts in the plasma fraction compared to packed blood cells (B. Hoffmann, unpublished observations). BTV has a high affinity to RBC of ruminants (Brewer and MacLachlan 1992, Brewer and MacLachlan 1994). Differences in the camelid erythrocyte morphology and structure (Omorphos *et al*. 1989) could influence the binding capacity and thus explain the resulting weak viral genome load of the cellular fraction of SAC blood observed both *in vivo* and *in vitro* (Fig. 22 and Fig. 23).

4. Bluetongue vaccination

4.1. Vaccination study

Bluetongue vaccination of more than 80% of the susceptible ruminant population in BTV-infected areas significantly reduces virus circulation and the risk of spreading the infection when moving animals to BTV-free areas (Giovannini *et al*. 2004b). SAC are susceptible to BTV infection (Rivera *et al*. 1987, Mattson 1994, Afshar *et al*. 1995a, Fowler 1998c, Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b, Schulz *et al*. 2012b), but their role in BTV epidemiology remains unknown. To minimize the risk of BTV spread and virus circulation, vaccination of SAC should therefore be considered in vaccination campaigns. Inactivated BTV-8 vaccines were developed for domestic ruminants, and were successfully used in mandatory vaccination programs (Gethmann *et al*. 2010). However, manufacturers did not give recommendations for SAC, and no information about immunogenicity and safety of any BTV vaccine was available. Therefore, doses for the vaccines used in this study were chosen by the vaccinating veterinarians. We had no influence on the vaccines and doses used in the study resulting in a relatively low number of animals in some study groups. However, a general trend can be evaluated.

All SAC that were vaccinated twice seroconverted, while a lower seroconversion rate was found in animals that were vaccinated with a single dose (Table 12). On the other hand, three SAC were seronegative three weeks after the 2nd vaccination, but were positive in the next sample taken 6 months p.v. In two of those animals, a slight humoral immune response below the cut-off value of the ELISA had already been detected in the three-week sample (data not shown). In contrast, cattle vaccinated twice (same three vaccines as used in this study) showed no obvious differences in seroconversion rates three weeks (Gethmann *et al*. 2009) or one year (Wäckerlin *et al*. 2010) after vaccination. However, lower seroconversion rates were recorded for SAC (52%) (Zanolari *et al*. 2010a) and ruminants (29 to 100%) (Gethmann *et al*. 2009) three weeks after the 1st vaccination, just as for sheep one year after a single vaccination compared to sheep vaccinated twice (Wäckerlin *et al*. 2010). On the other hand, those surveys were conducted with a cELISA (VMRD) that is considered less sensitive for antibodies in vaccinated animals compared to DR ELISAs (Eschbaumer *et al*. 2009, Oura *et al*. 2009, Oura *et al*. 2010). In the present study, no serum samples were collected between vaccinations, however, significantly lower seroconversion rates were found with the same cELISA (VMRD) than with the DR ELISA four weeks after the 2nd vaccination of SAC. Therefore, the results are not directly comparable.

Similar to the results of our study, Wäckerlin *et al.* (Wäckerlin *et al*. 2010) observed a significantly lower variance of antibody levels in sheep vaccinated twice compared to animals that received a single dose of an inactivated BTV-8 vaccine. The delayed or reduced seroconversion and heterogeneity in antibody levels can possibly be explained by a reduced responsiveness to vaccination of individual animals as observed in a llama by Zanolari *et al.* (2010a) and in several studies with ruminants (Oura *et al*. 2009, Eschbaumer *et al*. 2009). Bartram *et al.* (2010) suggested that immunogenicity is influenced by differences in the vaccine formulation. Furthermore, our data indicate that alpacas and llamas require a higher dose of BTV-8 vaccine per kilogram of body weight than sheep and cattle for a high seroconversion rate and homogeneous antibody levels. The use of higher doses in SAC to ensure successful treatment was already recommended for other drugs (Zanolari *et al*. 2008). Accordingly, for initial immunization we recommend to administer a minimum dose of twice 2 ml of vaccine to obtain a solid and homogenous serological response.

The likelihood of a distortion of the data due to vectorborne introduction of BTV in the study herds after BTV-8 vaccination is considered low. The vaccine study was performed in regions with low risk of BTV infection (Conraths *et al*. 2009, EU-BTNET system 2009), and a sudden increase in antibody levels that could have indicated a natural infection during the study was not recorded. A marked increase in the antibody level was observed in BTV-8 vaccinated ruminants after challenge infection with live virus (Wäckerlin *et al*. 2010) and in a llama that had been seropositive before vaccination (data not shown). Controls were only available in some herds, and all remained seronegative. Vaccinations of the study animals without our knowledge are very unlikely as we stayed in close contact with the owners during the whole study period and the owners were well-informed and called upon to report if the study animals were vaccinated again.

4.2. Tolerance of BTV-8 vaccines

In general, no or only mild adverse effects have been observed for the used vaccines and doses in most SAC. This is in line with the findings for SAC (Zanolari *et al*. 2010a) and ruminants reported by other groups (Gethmann *et al*. 2009, Bruckner *et al*. 2009b, Tschuor *et al*. 2010, Vitour *et al*. 2011, Bréard *et al*. 2011). Booster vaccination of SAC in subsequent years was well tolerated and the reproduction rate did not change. However, no close surveillance other than observation of the animals by the owners was conducted and some adverse effects might have been missed. In neither of the two fatal cases a direct link to

vaccination was identified, but in the case of the alpaca male a type I (immediate) hypersensitivity reaction to vaccination cannot be excluded.

4.3. Colostral BTV-8 antibody transfer

Interference of colostral immunity with the induction of an immune response to vaccination during the refractory period was described for SAC and ruminants by several groups (Ernst and Butler 1983, Wernery and Kaaden 2002b, Savini *et al*. 2004a, Oura *et al*. 2010, Vitour *et al*. 2011). Thus, for a successful vaccination regimen it is fundamental to know how long colostral immunity persists (Savini *et al*. 2004a). The transfer of colostrum-derived BTV antibodies to crias born from vaccinated dams was documented here for the first time.

No correlation was found between the antibody levels of the BTV-8 vaccinated dams and their crias measured by ELISA (Fig. 26). A possible explanation is that the uptake of immunoglobulins by newborns is influenced by various other factors such as the time and extent of colostrum intake p.n. (Dominguez *et al*. 2001, Wernery 2001). This is illustrated by two crias (7 and 8) that have been born from the same dam in consecutive years (Fig. 25). The antibody level of the mother remained stable within the year, but the antibody levels found in the two crias at 13 and 2 weeks p.n. diverged considerably.

The progression and persistence of detectable BTV antibodies in crias obviously depended on the initial antibody level. A similar parallel decrease in maternal antibody levels has been found in juvenile ruminants (Wernery 2001, Dominguez *et al*. 2001, Vitour *et al*. 2011) and in crias (Weaver *et al*. 2000, Wernery 2001) - especially in animals with a high initial level. The median interval p.n. for crias to become negative in the ELISA was similar (>14 to 26 weeks) to that reported for calves (16 to 25 weeks, depending on the cut-off value) (Vitour *et al*. 2011). In contrast, NAbs have only been detected in one cria (9) at six weeks p.n., while NAbs were detectable in calves (Vitour *et al*. 2011) and lambs (Oura *et al*. 2010) for over 10 weeks p.n. Furthermore, no correlation has been found between antibody levels measured by ELISA and SNT in dams and crias (Table 13). This was also observed in calves with colostral BTV antibodies depending on the time p.n. (Vitour *et al*. 2011). A possible reason is that the ELISA and the SNT target distinct antibody populations: antibodies to the core protein VP7 and the outer capsid proteins VP2 and VP5, respectively (Huismans and Erasmus 1981, Vitour *et al*. 2011). Possible explanations why this particular cria had NAbs p.n. are that (i) the cria was born considerably earlier (3 months) after the vaccination of the dam compared to the other crias (5 months), (ii) the dam had the highest NAb titre around parturition, and (iii) only this dam was vaccinated with vaccine F. Differences in the vaccine formulations

(Bartram *et al*. 2010) together with an ineffective antigen stimulation (Savini *et al*. 2009) were suggested to have an influence on the production of different antibody populations and, therefore, their transfer to newborns (Vitour *et al*. 2011). Booster vaccination of females shortly before parturition to ensure a maximum level of specific immunoglobulin G in the colostrum is recommended for SAC (Wernery and Kaaden 2002b, Fowler and Bravo 2010) and for ruminants (Doll 2006). In a recent study, NAbs were found in only a few lambs from ewes vaccinated once with an inactivated BTV-8 vaccine, but in all lambs from ewes vaccinated twice (Oura *et al*. 2010). Therefore, the amount of neutralising BTV-8 antibodies transferred to neonates in the present study was probably influenced by the dosage and the type of BTV-8 vaccine used as well as the time of vaccination of the dams before parturition. However, a higher number of animals are required to properly evaluate this observation for inactivated BTV-8 vaccines in SAC.

Oura *et al.* (2010) and Vitour *et al.* (Vitour *et al*. 2011) reported interference of colostrum-derived BTV-8 NA with antibody production after challenge and vaccination of lambs and calves, respectively. Failure of passive antibody transfer occurs regularly in neonates (Weaver *et al*. 2000, Vitour *et al*. 2011). Thus, it was recommended to vaccinate calves (Vitour *et al*. 2011) and lambs (Alpar *et al*. 2009) around the time of weaning (5 to >6 months p.n.) when outside the high-risk season of BTV transmission. During the vector season, however, they should be vaccinated as early as possible and receive a booster vaccination around the age of six months (Alpar *et al*. 2009, Vitour *et al*. 2011). Similarly, it appears reasonable to vaccinate crias late or twice around the time of weaning, especially those born from dams that had been vaccinated shortly before parturition (Wernery and Kaaden 2002b, Fowler and Bravo 2010).

In ruminants, NAbs are generally considered protective against viraemia and clinical disease after challenge with the homologous BTV serotype (Savini *et al*. 2008). However, the DR ELISA is also considered an appropriate means of verifying successful vaccination against BTV-8 (Eschbaumer *et al*. 2009, Oura *et al*. 2009). In this study, NAbs were only analysed for a few SAC, and we cannot exclude that an animal that is negative in the DR ELISA has a protective NAb response (Oura *et al*. 2009). We considered SNT as a possible confounder of the results obtained by DR ELISA due to the following reasons. A challenge infection was not conducted in the present study, but protective immunity has been reported for BTV-vaccinated ruminants (Savini *et al*. 2004b, Eschbaumer *et al*. 2009, Savini *et al*. 2009) and for some lambs with colostrum-derived BTV antibodies (Oura *et al*. 2010) that were positive in an ELISA but negative for NAbs. The BTV SNT has a considerably lower

sensitivity, even in naturally infected animals (Savini *et al*. 2004d, Bulut *et al*. 2006). A high proportion of false negative results in the SNT were reported for animals exposed to the Northern European BTV-8 strain, particularly after vaccination and experimental infection (Worwa *et al*. 2012). Furthermore, animals negative by SNT can still be protected against challenge infection (Eschbaumer *et al*. 2009). To date, there are no reports of animals that had been negative in a DR ELISA after vaccination with an inactivated BTV-8 vaccine but were still protected against challenge infection.

VII. COLLECTIVE DISCUSSION

BT is a major disease of ruminant livestock that can cause substantial impact on income and animal welfare (Wilson and Mellor 2009). In general, data on the susceptibility of SAC to BTV infection were sparsely documented. BTV-related fatalities in a few SAC during the recent BTV-8 and BTV-1 epizootics in Germany (Henrich *et al*. 2007) and France (Meyer *et al*. 2009), respectively, raised concern about the role of camelids in the epidemiology of BTV. This prompted the investigation of BTV-8 infection in SAC and the *Culicoides* fauna in SAC herds in Germany and a vaccination study to monitor antibody development and tolerance to BTV-8 vaccines in SAC and their offspring.

This is the first entomological monitoring conducted on SAC farms. The obtained results revealed that a high number of *Culicoides* spp. that have been incriminated as potential vectors of BTV in ruminant herds also occur on SAC farms in Germany. The higher incidence of BTV vectors at the Hessian farm together with various local factors that can positively influence the abundance of *Culicoides* (Mayo *et al*. 2012a) are possible reasons for the higher BTV seroprevalence found in this herd.

Analysis of risk factors collected in the cross-sectional study demonstrated that the risk for BTV infection in a SAC population was only associated with BTV exposure, pressure of infection and probably with age. However, additional investigations with BTV-8 and other BTV serotypes are necessary to generalise these observations. Higher infection rates in regions where BTV outbreaks have occurred first and an association between BTV infection and age have also been reported by other groups (Ward *et al*. 1994, Conraths *et al*. 2009, Linden *et al*. 2010, Durand *et al*. 2010b, Falconi *et al*. 2011).

Furthermore, we have demonstrated for the first time that SAC can be experimentally infected with BTV-8. No differences were found between llamas and alpacas concerning clinical signs and serological and virological analyses. After BTV infection, the humoral immune response

of SAC was similar to that of domestic and wild ruminants, camels and llamas (Foster *et al*. 1991, Afshar *et al*. 1995a, Darpel *et al*. 2007, Eschbaumer *et al*. 2009, Dal Pozzo *et al*. 2009a, López-Olvera *et al*. 2010, Eschbaumer *et al*. 2010b, Batten *et al*. 2011). Viral RNA, on the other hand, was detected by RT-qPCR for only 28 to 35 dpi (Schulz *et al*. 2012b), which contrasts the long-term viraemia characteristic for BTV infection in domestic and wild ruminants (MacLachlan *et al*. 2009, López-Olvera *et al*. 2010). Similar to SAC, C_q values in camels experimentally infected with BTV-1 were at the detection limit. However, BTV RNA was detected several days later and for a longer period (up to 68 dpi) than in SAC. As an exceptional result, infectivity of BTV in SAC could only be demonstrated by inoculation of highly susceptible IFNAR$^{-/-}$ mice, but not with methods regularly used for BTV isolation from bovine blood (see II.4) (Verwoerd and Erasmus 2004, Eschbaumer *et al*. 2010a). In contrast, virus was successfully isolated from dromedary blood on KC-cells (Batten *et al*. 2011).

The blood-cell binding experiment revealed for the first time that BTV-8 adsorbs less efficiently to SAC erythrocytes than to bovine RBC, which might be the reason for the different progression of viraemia in BTV-infected SAC (Schulz *et al*. 2012b). In contrast to ruminants (Verwoerd and Erasmus 2004), whole blood, serum or plasma should be used for BTV detection in SAC blood, but not blood cell pellets (Schulz *et al*. 2012b). What is more, the samples obtained from the animal experiment allowed the validation of various virological and serological test systems for BTV diagnosis in SAC.

The results of the animal experiment together with those of the cross-sectional study provide further evidence for the susceptibility of SAC to BTV (Schulz *et al*. 2012b, 2012c). In addition, the presented BTV-related cases together with those reported by other groups (Henrich *et al*. 2007, Meyer *et al*. 2009, Ortega *et al*. 2010b) have demonstrated that the susceptibility of SAC to BT disease has to be reconsidered. However, none of the 249 seropositive animals showed obvious clinical signs of disease on the study farms, and the clinical signs in the experimentally infected SAC were unspecific and mild. Therefore, this disease is obviously of minor importance for SAC, even if sporadic cases may occur. On the other hand, mild and unspecific clinical signs, such as those observed after experimental BTV infection of SAC, may be missed or misinterpreted by owners and veterinarians in the field.

The vaccination study revealed that for initial immunisation with an inactivated BTV-8 vaccine SAC should be vaccinated twice at an interval of 3 to 4 weeks to obtain a solid and homogenous humoral immune response that persists for at least one year p.v. Adequate antibody development was demonstrated for the first time for BLUEVAC®-8 and Zulvac® 8 Bovis in SAC. A higher seroprevalence and a more homogenous antibody response for at

least one year in animals that received a booster vaccination were also shown by Wäckerlin *et al*. (2010). Good tolerance for the used vaccines were in accordance with results obtained after BTV-8 vaccination with inactivated vaccines of SAC (Zanolari *et al*. 2010a) as well as of domestic and wild ruminants (Gethmann *et al*. 2009, 2010, Probst *et al*. 2011, Lorca-Oró *et al*. 2012).

The transfer of maternal BTV antibodies to crias born from BTV-vaccinated dams was demonstrated for the first time. To avoid failure of passive antibody transfer due to interference of colostral immunity with the induction of an immune response, it appears reasonable to vaccinate crias late after colostrum-derived antibodies have disappeared or at least considerably waned (~6 months p.n.) (Ernst and Butler 1983, Wernery and Kaaden 2002b, Savini *et al*. 2004a, Alpar *et al*. 2009, Oura *et al*. 2010, Vitour *et al*. 2011). Late vaccination p.n. is specifically applicable to crias from dams that were immunised shortly before parturition, to induce a high level of specific BTV antibodies (Wernery and Kaaden 2002b, Doll 2006, Fowler and Bravo 2010). However, early vaccination followed by a booster vaccination at an age of about six months should be implemented during the vector season (Alpar *et al*. 2009, Vitour *et al*. 2011).

VIII. CONCLUSIONS AND OUTLOOK

The negative virological results obtained by the cross-sectional study and the entomological monitoring - despite of the on-going BTV-8 - epizootic can be explained by the low amounts of virus occurring only for a short time in SAC blood. On the other hand, the cross-sectional study and the entomological monitoring were conducted after the peak of the BTV-8 epizootic in Germany in 2007 (Conraths *et al*. 2009), reducing the chance to find BTV-positive samples. The animal experiment revealed remarkable differences in the pathogenesis of BTV infection in SAC. The low amounts of BTV RNA detected for a short time in SAC blood contrasted the long-term viraemia characteristic for BTV infection in ruminants. Therefore, these three studies indicated that SAC play no important role in the epidemiology of BTV. Further research will be necessary to evaluate the capacity of SAC as a source of infection for *Culicoides* midges taking a blood meal in the viraemic period. Investigation of breeding habitats of *Culicoides* on SAC farms would contribute to improve targeted vector control (Werner *et al*. 2012).

The vaccination study provides vaccination recommendations to initiate an appropriate BTV-8 antibody response in SAC. Challenge experiments are needed to evaluate the protective

immunity in BTV-vaccinated SAC and their offspring during the passive immunity. Of particularly interest would be to assess whether passive immunity in crias interferes with the induction of an immune response to BTV infection or BTV vaccination.

Compared to other mammals, camelids have an exceptional immune system (Omorphos *et al*. 1989, Wernery *et al*. 1999, Wernery 2001, Conrath *et al*. 2003, Wernery and Kaaden 2004). The susceptibility of SAC to important animal diseases like foot-and-mouth disease (reviewed by Wernery and Kaaden 2004) and bovine herpesvirus 1 infection (P. König, unpublished observations) is much reduced, and they only play a minor or negligible role as carriers. Based on the results of the presented work, SAC play a negligible role in the epidemiology of BTV. In light of the phylogenetic differences between camelids and ruminants, SAC should be considered separately when policy decisions in epidemiology and disease control are made.

IX. SUMMARY

Bluetongue (BT) is a *Culicoides*-born infectious disease caused by bluetongue virus (BTV). From 2006 to 2010, BTV serotype 8 (BTV-8) spread throughout Europe, causing severe disease in domestic and some wild ruminant species and in an alpaca. Compulsory vaccination of susceptible animals was the most effective strategy to control and eradicate the BTV-8 epizootic in Europe. However, South American camelids (SAC) were not included in the BTV-8 vaccination programmes in Europe.

The presented work elucidates the potential role of SAC in the epidemiology of BTV as reservoirs and risk for the ruminant population as well as the outcome of BTV-8 vaccination in SAC. The investigation of BTV-8 vectors, pathogenesis, epizootiology, diagnosis and immunoprophylaxis in SAC herds comprised four approaches:

The entomological monitoring on SAC farms in Germany from 2008 to 2009 revealed a significantly high abundance of *Culicoides* belonging to the *C. obsoletus* and *C. pulicaris* complexes - potential vectors of BTV. At the same time, a high seroprevalence was found in BTV-8 exposed SAC herds in Germany. However, no BTV RNA was detected in the analysed *Culicoides* midges and SAC blood although the BTV-8 epizootic was still going on. The unexpected virological results of these two studies prompted the investigation of BTV pathogenesis in SAC.

Experimental infection of SAC with BTV-8 revealed considerable differences in their pathogenesis compared to ruminants. SAC displayed only very mild, unspecific clinical signs. BTV genome load was significantly lower than in bovine blood, was only transiently detected in SAC blood and rapidly declined after seroconversion. This matched the results of the blood-cell binding experiment, which demonstrated that significantly lower amounts of BTV-8 adsorbed to SAC blood cells compared to bovine blood cells. Therefore, whole blood, serum or plasma should be used for BTV diagnosis in SAC, but not blood cell pellets.

Within the scope of this work, available serological and virological test systems were validated for BTV diagnosis in SAC. The crucial outcome of these three studies was the virological results indicating that SAC obviously play a negligible role in the epidemiology of this virus infection. Furthermore, BT is apparently not an important disease of SAC although fatalities may sporadically occur.

The vaccination study demonstrated for the first time that SAC vaccinated twice with any one of the three inactivated BLUEVAC®-8, BTVPUR® AlSap 8 and Zulvac® 8 Bovis

vaccines remain BTV-seropositive for over one year. However, seroconversion was only found in 79% of SAC vaccinated with a single dose. Furthermore, this is the first study demonstrating that colostral BTV-8 antibodies occur in crias from vaccinated dams for 2 to 7 months after birth. Good tolerance of the studied vaccines was reported retrospectively by SAC owners. This work provides vaccination recommendations to initiate an appropriate BTV-8 antibody response in SAC.

The new insights obtained by the presented work provide an important basis for further research on particularities of BTV infection in SAC and on their protective immunity after BTV-vaccination. Additionally, these results will facilitate risk assessment and the design of policies for the control of BT in SAC herds in case of a BTV outbreak in the future.

X. ZUSAMMENFASSUNG

Die Blauzungenkrankheit (*engl.* Bluetongue, BT) ist eine von *Culicoides* übertragene Infektionskrankheit, welche durch das Blauzungenvirus (*engl.* bluetongue virus, BTV) verursacht wird. Das BTV Serotyp 8 (BTV-8) breitete sich in den Jahren 2006 bis 2010 über ganz Europa aus. Dabei verursachte dieses Virus schwerwiegende Erkrankungen bei Haus- und einigen Wildwiederkäuerarten sowie bei einem Alpaka. Die Pflichtimpfung empfänglicher Tiere erwies sich als die wirksamste Strategie diese Tierseuche zu bekämpfen. Allerdings wurden Neuweltkameliden (NWK) nicht in die Impfprogramme einbezogen.

Die vorliegende Arbeit gibt Aufschluss über die potentielle Rolle von NWK in der BTV-Epidemiologie als Reservoire und Risikofaktoren für Wiederkäuerpopulationen und über die Wirkung von BTV-8-Impfungstoffen bei NWK. Die Untersuchungen zu Vektoren, Pathogenese, Epizootiologie und Diagnose von BTV-8 sowie die Immunprophylaxe gegen BTV-8 in NWK-Herden umfasste vier Herangehensweisen.

Das entomologische Monitoring auf deutschen NWK-Betrieben in den Jahren 2008 und 2009 ergab eine signifikant hohe Abundanz von den Komplexen *C. obsoletus* und *C. pulicaris* angehörigen *Culicoides*, welche potentiell BTV-Vektoren sind. Zur gleichen Zeit wurde eine hohe Seroprävalenz in BTV-8 exponierten NWK-Herden in Deutschland festgestellt, obwohl die Tierseuche immer noch andauerte. Aufgrund der unerwarteten virologischen Ergebnisse dieser zwei Studien wurden Untersuchungen zur Pathogenese BTV-infizierter NWK durchgeführt.

In einem Tierversuch zeigten sich beträchtliche Unterschiede zwischen der Pathogenese BTV-8-infizierter NWK und jener der Hauswiederkäuer. Bei NWK traten nur sehr milde, unspezifische klinische Symptome auf. Die BTV-Genomlast war signifikant geringer als in Rinderblut, wurde nur vorübergehend in NWK-Blut festgestellt und nahm nach der Serokonversion schnell ab. Dies stimmte mit den Ergebnissen des Blutzellbindungsexperiments überein, mit welchem demonstriert wurde, dass signifikant weniger BTV-8 an Blutzellen von NWK als an jene von Rindern adsorbierten. Aus diesem Grund sollte Serum oder Plasma für die BTV-Diagnose bei NWK verwendet werden, jedoch keine Blutzellen.

Im Rahmen dieser Arbeit wurden serologische und virologische Testsysteme für die Diagnose einer BTV-Infektion bei NWK validiert. Die entscheidendste Erkenntnis wurde anhand der virologischen Ergebnisse dieser drei Studien gewonnen. Diese wiesen darauf hin,

dass NWK in der Epidemiologie dieser Virusinfektion eine vernachlässigbare Rolle spielen. Zudem ist BT offensichtlich keine bedeutsame Erkrankung für NWK, auch wenn sporadische Todesfälle auftreten können. Weitere Studien sind erforderlich, um die Möglichkeit einer BTV-Infektion von *Culicoides*-Vektorspezies zu untersuchen, die Blut virämischer NWK aufnehmen.

In der Impfstoffstudie wurde erstmals demonstriert, dass NWK nach zweifacher Impfung mit einer der drei inaktivierten BLUEVAC®-8, BTVPUR® AlSap 8 and Zulvac® 8 Bovis Impfstoffe für die Dauer von über einem Jahr BTV-seropositiv bleiben. Dagegen war die Serokonversionsrate bei NWK, die nur eine Impfstoffdosis erhielten, deutlich geringer (79%). Des Weiteren wurden erstmals kolostrale BTV-8-Antikörper bei Fohlen von geimpften Muttertieren 2 bis 7 Monate nach der Geburt nachgewiesen. In einer retrospektiven Umfrage berichteten NWK-Besitzern eine gute Verträglichkeit der untersuchten BTV-8-Impfstoffe. Die meisten NWK-Besitzer berichteten retrospektiv eine gute Verträglichkeit der untersuchten BTV-8-Impfstoffe. Diese Arbeit liefert Impfempfehlungen, um eine angemessene BTV-8-Antikörperantwort bei NWK zu induzieren.

Die neu gewonnen Erkenntnisse aus der vorliegenden Arbeit liefern eine wichtige Grundlage für weitere Untersuchungen zu den Besonderheiten der BTV-Infektion bei NWK und deren schützende Immunität nach einer BTV-Impfung. Zudem werden die Ergebnisse eine Risikobewertung und den Entwurf von Richtlinien für die BTV-Bekämpfung in NWK-Herden im Falle eines BTV-Ausbruches in Zukunft erleichtern.

XI. APPENDIX

Appendix 1

Table 1. The genome segments and proteins of *Bluetongue virus* (Mertens *et al*. 2004, Verwoerd and Erasmus 2004, Schwartz-Cornil *et al*. 2008, Mertens and Attoui 2009, Noad and Roy 2009, Ratinier *et al*. 2011).

Genome segment	Encoded protein ([a])	Location (number of copies/virion)	Properties and functions
S1	VP1 (Pol)	Subcore (12)	Minor core protein, component of transcriptase complex: RNA-dependent RNA-polymerase, RNA synthesis
S2	VP2	Outer capsid (180)	Trimer, serotype-specific antigen, contains neutralizing epitopes, most variable protein, involved in determination of virulence, cell attachment, strong affinity for glycophorin A of erythrocytes
S3	VP3 (T2)	Inner (subcore) capsid (120)	Major core protein, decamer, interacts with subcore proteins, self-assembles, controls size and organization of capsid structure, RNA-binding, scaffold for VP7 layer
S4	VP4 (CaP)	Subcore (24)	Minor core protein, dimer, component of transcriptase complex: capping enzyme
S5	NS1 (TuP)	Infected cell	Non-structural protein in host cell cytoplasma, forms virus-specific tubules, unknown function, probably has a role in BTV cythopathogenesis
S6	VP5	Inner layer of the outer capsid (360)	Trimer, co-determinant of serotype, cytotoxic, mediates release of viral particles from endosome into cytoplasm
S7	VP7 (T13)	Core capsid (780)	Major core protein, serogroup-specific antigen, major immunodominant protein, can mediate attachment and penetration of insect cells
S8	NS2 (ViP)	Infected cell	Non-structural phosphoprotein in host cell cytoplasma, forms viral inclusion bodies, ssRNA binding and condensation, recruits VP3
S9	VP6 (Hel)	Subcore (72)	Minor core protein, component of transcriptase complex: ssRNA and dsRNA binding, helicase, NTPase
S9 (open reading frame)	NS4	Infected cell	Non-structural protein in nucleoli of host cell, important role in virus-host interaction and is one of the mechanisms played (at least by BTV-8) to counteract the antiviral response of the host
S10	NS3 NS3A	Infected cell	Non-structural glycoproteins, membrane proteins, involved in virus release from host cell: budding (insect and mammalian cells) and viroporin (more prominent in mammalian cells) mechanisms

[a] Protein structure/function: Pol, RNA polymerase; CaP, capping enzyme; Hel, helicase enzyme; T2, internal protein with T=2 symmetry; T13, internal protein with T=13 symmetry; ViP, viral inclusion body matrix protein; TuP, tubule protein; VP, viral protein; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.

Appendix 2

Table 1. Overview of vaccination campaigns against BTV-8 in Europe 2008 to 2011 (according to references [a] and [b]). Most countries in Europe have reverted to voluntary vaccination campaigns in 2011 (Oura 2011).

Country	Species	Vaccination coverage	Type of programme			
			2008[a]	2009[a]	2010[b]	2011[b]
Austria	B, S, G	>78%	mandatory	mandatory, voluntary as of July 2009[d]	voluntary	voluntary
Belgium	B, S, G, C	89%, 129% (B, S), 19% (G)	mandatory (B, S), voluntary (G, C)	mandatory (B, S), voluntary (G, C)	mandatory	voluntary
Czech Republic	B, S, G	84.4% (B), 93% (S), 93% (G)	?	mandatory	mandatory	?
Denmark	B, S, G	>86%, 2009: 76%[c] (B), 50% (S/G)	mandatory (not whole DK)	mandatory (not whole DK), exceptions[e]	voluntary	prohibited (free since 1 Jan. 2011)
France	B, S, G	2008: low 2009: <50% to >90% (B > S/G)	voluntary	mandatory	mandatory	voluntary
Germany	B, S, G	>80%	mandatory	mandatory	voluntary	voluntary
Italy	B, Bf, S, G		mandatory (restriction zone)	mandatory (restriction zone)	mandatory (restriction zones?), Sardinia: voluntary[f]	?
Netherlands	B, S, G		voluntary	voluntary	voluntary	voluntary
Portugal	B, S, G		voluntary	voluntary	voluntary (cattle) mandatory (sheep)	voluntary
Sweden	B, S, G	>80%	mandatory (southern SE)	mandatory (southern SE)	mandatory: southern SE	?
Switzerland	B, S, G	>90%	mandatory	mandatory	mandatory	voluntary
Spain	B, S	about 70%	mandatory	mandatory	mandatory	voluntary
UK	B, S, G	26% to 95% (England and Wales) Scotland: high	voluntary (England, Wales) mandatory (Scotland)	voluntary (England, Wales) mandatory (Scotland)	voluntary (lower risk zone since 12 June 2010)	?

Appendix 2, Table 1 continued

Sources used to gather information on vaccination campaigns conducted 2010–2011 by country.

Country	Source (URL)
Austria	http://www.agrarnet.info/?id=2500%2C1462557%2C%2C
Belgium	http://www.vetsweb.com/news/bluetongue-vaccination-rules-change-in-belgium-2007.html
Czech Republic	http://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra10_7.pdf
Denmark	http://www.uk.foedevarestyrelsen.dk/AnimalHealth/Bluetongue/forside.htm
France	http://www.fcoinfo.fr/spip.php?article485
Germany	http://www.bmelv.de/SharedDocs/Standardartikel/Landwirtschaft/Tier/Tiergesundheit/Blauzungenkrankheit/Blauzungenkrankheit-Impfung.html
Italy	http://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra10_7.pdf
	http://www.adnkronos.com/IGN/Regioni/Sardegna/Lingua-blu-vaccinazione-solo-per-animali-da-esportare_4074917812.html
Netherlands	http://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra10_7.pdf, 2011: Personal communicationJGethmann
Portugal	http://www.apcrf.pt/fotos/editor2/edital27.pdf
	http://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra10_7.pdf
Sweden	http://www2.jordbruksverket.se/webdav/files/SJV/trycksaker/Pdf_rapporter/ra10_7.pdf
Switzerland	http://www.bvet.admin.ch/gesundheit_tiere/01973/index.html?lang=de
Spain	http://www.oviespaña.com/mercados.shtml?idboletin=1707&idseccion=9252&idarticulo=80119
UK	http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/bluetongue/control/vaccination/index.htm
	http://www.scotland.gov.uk/Topics/farmingrural/Agriculture/animal-welfare/Diseases/disease/Bluetongue/control

[a] References for 2008–2009 retrieved from FLI 2009, Gethmann *et al*. 2010 ; [b] References for the years 2010–2011 are given in column "Source (URL)"; [c] in addition to animals vaccinated in 2008; [d] http://www.agrarnet.info; [e] e.g., for herds <10 animals; [f] mandatory animals for exportation; B, bovine; S, sheep; G, goats; C, cervids; Bf, buffalo

Appendix 3

Table 1. Field studies of BTV infection in camelids.

Country	Species	Method	Target	BTV serotype	Clinical observation	No. tested	Prevalence (%)	Reference
SOUTH AMERICAN CAMELIDS:								
South America								
Argentina (Chubut)	Guanacos	ELISA, AGID	Abs			20	0	(Karesh *et al*. 1998)
	Sheep		Abs			20	0	
(Buenos Aires, Cordoba, Jujuy)	Llamas	AGID	Abs			390	0	(Puntel *et al*. 1999)
	Cattle, sheep		Abs			146, 30	0	
(Jujuy)	Vicuñas, llamas	ELISA	Abs			128, 17	0, 0	(Marcoppido *et al*. 2010)
	Cattle		Abs			15	0	
(Río Negro → Buenos Aires)	Guanacos	AGID	Abs			11	0	(Marcoppido *et al*. 2011)
Peru	Alpacas	AGID	Abs		None	114	21	(Rivera *et al*. 1987)
North America								
USA (Oregon)	Llamas	-	Abs		None	270	1.5	(Picton 1993, cited by Mattson 1994)
Europe								
France (Crampagna, Ariège)	Llamas	ELISA	Abs		None	9	78	(Meyer *et al*. 2009)
		RT-qPCR	RNA	BTV-1			78	
	Llamas	RT-qPCR	RNA	BTV-1	None	18	50	
	Llamas	RT-qPCR	RNA	BTV-1	None	-	60	
	Llamas	-	-	BTV-8	None	-	-	
Germany	Alpacas (and Llamas)	ELISA	Abs	BTV-8	None	93	1	(Locher *et al*. 2010)
Switzerland	Llamas	ELISA	Abs			159	0	(Zanolari *et al*. 2010b)
	Alpacas	ELISA	Abs			195	0	

Appendix 3, Table 1 continued

Country	Species	Method	Target	BTV serotype	Clinical observation	No. tested	Prevalence (%)	Reference
OLD WORLD CAMELIDS:								
Africa								
Algeria	Camels, cattle, goats, sheep	ELISA	Abs			92, 852, 71, 359	21, 29, 21, 14	(Madani *et al*. 2011)
	Camels, cattle, goats, sheep	RT-PCR	RNA	BTV-1		19, 250, 15, 51	0, 13.6, 0, 5.9	
Botswana	Camels	-	Abs			-	81	(Simpson 1979)
Egypt	Camels	-	Abs			-	14.3	(Hafez and Ozawa 1973, cited by Wernery and Kaaden 2002a)
Morocco	Camels	ELISA	Abs			500	32 to 87	(Batten *et al*. 2011)
(in 2003)	Dromedaries	ELISA	Abs			536	11.0	(Touil *et al*. 2012)
(in 2009)	Dromedaries	ELISA	Abs			836	25.6	(Touil *et al*. 2012)
Sudan (Tambool)	Camels	AGID	Abs			102	4.9	(Eisa *et al*. 1979, 1980)
Sudan	Cattle, goats, sheep	AGID	Abs			874, 98, 980	8, 11.2, 28	(Eisa *et al*. 1979, 1980)
	Camels, cattle, sheep	AGID	Abs	BTV-4		89, 40, 73	14.6, 75, 80	(Abu Elzein 1984)
	Camels, cattle, sheep	AGID	Ag	BTV-4		89, 40, 73	5.6, 7.5, 16.3	
Near East								
Iran	Camels	-	Abs			-	5.9	(Afshar and Kayvanfar 1974)
	Cattle, goats, sheep					35, 647, 1081	11.2, 12.6, 5.5	
Israel	Camels	AGID	Abs			51	17.6	(Barzilai 1982)
	Camels	NT	Abs	BTV-4 (n=16)		37	48.6	
Saudi Arabia	Dromedaries	-	Abs			3	67	(Hafez and Ozawa 1973, cited by Wernery and Kaaden 2002a)
	Camels		Abs			-	58	(Ostrowski, in press, cited by Wernery *et al*. 2008)
	Dromedaries	AGID	Abs			493 (99 East)	1.2 (6 East)	(Al-Afaleq *et al*. 2007)

Appendix 3, Table 1 continued

Country	Species	Method	Target	BTV serotype	Clinical observation	No. tested	Prevalence (%)	Reference
OLD WORLD CAMELIDS:								
Near East continued								
United Arab Emirates	Camels	AGID	Abs			1023	< 1	(CVRL Annual Report 1998, cited by Wernery and Kaaden 2002a)
	Camels	ELISA	Abs			211	5	
	Sheep		Abs			-	35	
	Dromedaries	ELISA	Abs			1119	21	(Wernery *et al*. 2007)
Yemen (Hudaydah)	Camels	AGID	Abs			31	13	(Stanley 1990)
	Cattle, goats, sheep		Abs			712, 397, 735	15, 24, 21	
India								
Gujarat	Dromedaries	AGID	Abs			-	9.3	(Chandel and Kher 1999, cited by Prasad *et al*. 2009)
	Camels	AGID	Abs		Higher seropreva-lences associated with stiffness & trypanoso-miasis	176	12.5	(Chandel *et al*. 2003)
	Camels	ELISA	Abs			176	19.3	
	Dromedaries	AGID	Abs			326	26.69	(Chauhan *et al*. 2004)4[a]
	Dromedaries	ELISA	Abs			326	38.34	
	Dromedaries	NT	Abs	(BTV 1-4, 10, 12, 14-18, 20, 21, 24)		326	-	
	Camels	AGID	Abs		Higher seropreva-lences associated with dermatitis & stiffness	82	25.61	(Patel *et al*. 2007)
	Camels	CCIE	Abs			82	28.05	
	Camels	ELISA	Abs			82	37.80	
Rajasthan	Dromedaries	AGID	Abs				9.9	(Malik *et al*. 2002, cited by Prasad *et al*. 2009)

AGID, Agar gel immunodiffusion; CCIE, Counter current immunoelectrophoresis; ELISA, Enzyme linked immunosorbent assay; NT, Neutralisation test; RT-qPCR, quantitative real-time RT-PCR; Abs, Antibodies; Ag, Antigen; RNA, BTV RNA; Vi, virus isolation; none, no clinical signs observed; -, no data available;
[a] http://www.cababstractsplus.org/abstracts/Abstract.aspx?AcNo=20053065456

Table 2. Case reports and animal experiments on BTV infection in camelids.

Country	Species	Method	Target	BTV serotype	Clinical observation	No. tested	Type of study	Reference
SOUTH AMERICAN CAMELIDS:								
North America								
Canada	Llamas	cELISA	Abs	BTV-10	None	2	EI	(Afshar *et al*. 1995a)
USA	Llama	ELISA	Abs		Respiratory distress followed by ▼ & increase in Abs titre	1	CR	(Fowler 1998c)
USA (California)	Alpacas	RT-qPCR, sequence analysis	RNA	BTV	†	1	CR	(Ortega *et al*. 2010a)
Europe								
Germany (Hesse)	Alpaca	RT-qPCR	RNA	BTV-8	†	1	CR	(Henrich *et al*. 2007)
France (Auzat, Ariège)	Llama mare, foetus	RT-qPCR	RNA	BTV-1	▼	1	CR	(Meyer *et al*. 2009)
	Llama foetus	Vi on BHK-21	BTV	BTV-1				
	Llamas	RT-qPCR	RNA	BTV-1	†	2	CR	
		Vi on BHK-21	BTV	BTV-1				
OLD WORLD CAMELIDS:								
Africa								
Morocco	Dromedaries	cELISA	Abs	BTV-1	None	3	EI	(Batten *et al*. 2011)
		RT-qPCR	RNA	BTV-1				
		Vi on KC-cells	BTV	BTV-1				

cELISA, competitive Enzyme linked immunosorbent assay; NT, Neutralisation test; RT-qPCR, quantitative real-time RT-PCR; Vi, Virus isolation; BHK-21, Baby hamster kidney-21 cells; KC-cells, derived from *Culicoides sonorensis* embryos; $IFNAR^{-/-}$, interferon alpha/beta receptor deficient mouse; Abs, Antibodies; RNA, BTV RNA; Vi, virus isolation; None, No clinical signs observed; -, No data available; ▼, Abortion; †, fatal

Appendix 4

Table 1. BTV-8 seroprevalence in individual SAC and in SAC herds by federal state in Germany 2008/2009.

Region	Federal state	Individual SAC			Herds		
		No. of samples	No. positive	% (95% CI)	No. of samples	No. positive	% (95% CI)
West	Rhineland-Palatinate	37	20	54.1 (36.9-70.5)	3	2	66.7 (9.4-99.2)
	North Rhine-Westphalia	214	112	52.3 (45.4-59.2)	11	9	81.8 (48.2-97.8)
	Saarland	4	2	50.0 (6.8-93.2)	2	1	50.0 (12.6-98.7)
	Hesse	136	33	24.3 (17.3-32.4)	7	6	85.7 (42.1-99.6)
Northern-coastal	Schleswig-Holstein	64	9	14.1 (6.6-25.0)	4	3	75.0 (19.4-99.4)
	Lower Saxony	153	16	10.5 (6.1-16.4)	7	6	85.7 (42.1-99.6)
	Mecklenburg-Western Pomeranian	6	0	0.0 (0.0-45.9)	1	0	0.0 (0.0-97.5)
South	Baden-Wuerttemberg	413	47	11.4 (8.5-14.8)	19	12	63.2 (38.4-83.7)
	Bavaria	338	7	2.1 (0.8-4.2)	18	3	16.7 (3.6-41.4)
East	Saxony-Anhalt	34	3	8.8 (1.9-23.7)	3	1	33.3 (0.8-90.6)
	Saxony	145	0	0.0 (0.0-2.5)	9	0	0.0 (0.0-33.6)
	Thuringia	102	0	0.0 (0.0-3.5)	4	0	0.0 (0.0-60.2)
	Brandenburg	96	0	0.0 (0.0-3.8)	3	0	0.0 (0.0-70.8)
	Total	1742	249	14.3 (12.7-16.0)	91	43	47.3 (36.7-58.0)

95% CI, 95% confidence interval

Appendix 4 continued

Table 2. BTV-8 seroprevalence in SAC by species, age and sex in Germany 2008/2009.

SAC species and breeds	Sex and age groups[1]	Seropositive herds (n=43)			All herds (n=91)	
		No. of samples	No. positive	% (95% CI)	No. of samples	% (95% CI)
Huacayas[2]	Crias[3]	133	32	24.1 (17.1-32.2)	290	11.0 (7.7-15.2)
	Yearlings[3]	70	10	14.3 (7.1-24.7)	165	6.1 (2.9-10.9)
	Males	81	28	34.6 (24.3-46.0)	191	14.7 (10.0-20.5)
	Females	200	74	37.0 (30.3-44.1)	478	15.5 (12.4-19.0)
	Unknown	46	0	0.0 (0.0-7.7)	58	0.0 (0.0-6.2)
Suri	Crias	2	0	0.0 (0.0-84.2)	19	0.0 (0.0-17.6)
	Yearlings	0	0	0	9	0.0 (0.0-33.6)
	Males	2	0	0.0 (0.0-84.2)	12	0.0 (0.0-26.5)
	Females	6	2	33.3 (4.3-77.7)	27	7.4 (0.9-24.3)
All alpacas		540	146	27.0 (23.3-31.0)	1249	11.7 (10.0-13.6)
Llama	Crias[3]	73	18	24.7 (15.3-36.1)	98	18.4 (11.3-27.5)
	Yearlings[3]	28	8	28.6 (13.2-48.7)	37	21.6 (9.8-38.2)
	Males	82	25	30.5 (20.8-41.6)	115	21.7 (14.6-30.4)
	Females	167	44	26.3 (19.8-33.7)	226	19.5 (14.5-25.2)
	Unknown	0	0	0	3	0.0 (0.0-70.8)
All llamas		350	95	27.1 (22.6-32.1)	479	19.8 (16.4-23.7)
Huarizo	Crias	2	1	-	2	-
	Males	1	1	-	1	-
	Females	2	2	-	2	-
Guanaco hyb.	Geldings	3	2	-	3	-
	Females	2	1	-	3	-
Vicuñja hyb.	Males	1	1	-	1	-
Vicuñja	Males	0	0	0	1	-
	Females	0	0	0	1	-
All other breeds		11	8	72.7 (39.0-94.0)	14	57.1 (28.9-82.3)
All SAC		901	249	27.6 (24.7-30.7)	1742	14.3 (12.7-16.0)

[1] Crias, < 1 years of age (y); yearlings, > 1 y; adults, > 2 y; [2] Huacayas and 69 alpacas of unknown breed, [3] The sex ratio among crias and yearlings was about 1:1; hyb., hybrid

Appendix 5

Fragebogen Lamavereinsmitglieder:	**NAME:**		**DATUM:**	

1.	Adressänderungen		Adresse:									
			Tel:									
			Mobil:									
			Email- Adresse:									
			Homepage:									
2.	Betriebsname											
1.	Tiere (Arten, Rasse, Geschlecht, Anzahl)	Summe NWC										
			Ja	Anzahl	Stuten	Andere		Cria <1J		Haltung		
						W	H	F	M	Mix	Separat	
		Lamas	☐									
		Alpakas	☐									
		Suri	☐									
		Huacaya	☐									
		Guanako	☐									
		Trampelt	☐									
		Dromedar	☐									
		Schafe	☐									
		Ziegen	☐									
		Sonstige	☐									
			Rinder,									
			Pfd									
			Schwei									
			Hd									
			Ktz,etc									
		Sum	Alle									
			NWC									
2.	Nutzungsrichtungen:		Zucht☐	Trekking☐	Therapie☐	Hobby☐	Wolle ☐					
	Tieranzahl											
	Hauptnutzungsrichtung		☐	☐	☐	☐	☐					
	Sonstiges:		Landschafts pflege☐	Ausbildung ☐	☐	☐						
3.	Hauptberuflich, Nebenerwerblich Hobby		☐ ☐ ☐									
4.	Hektar Weideland											
5.	NWC-Haltung seit:											
6.	BTV-Fälle		Ja *	Nein	Anzahl.	* wann?						
		Erkrankung	☐	☐								
		Todesfälle	☐	☐								
		Seropositive	☐									
		Seronegativ	☐									
		PCR-positiv	☐									
		PCR-negativ	☐									

Appendix 5 continued

		Wie diagnostiziert?				
		Kommentar				
		- Erkrankte oder seropositove/-negative oder pcr-positive	Rasse:	**Lamas**	**Alpakas**	**Andere**
			Zahl:			
			Alter:			
		- Todesfälle:	Zahl:			
			Alter:			
7.	BTV-Impfung	Ja ☐ (durchgeführt)	Hersteller/ Impfstoff+ Chargennr.			
		Anzahl ges.:	Dosierung			
			Nebenwirkungen			
			Tiere	Rasse:	**Lama**	**Alpaka**	**Andere**
				Zahl			
				Alter			
			Todesfälle	Zahl			
				Alter			
		Nein ☐				
		Impfung geplant	Ja ☐			
			Nein ☐			
			Wann			
			Wieviele			
			Tiere	Rasse	**Lama**	**Alpaka**	**Andere**
				Zahl			
				Alter			
8.	Zustimmung für eventuelle **Bestandsvisite** in Herbst/ Winter 2008 (Blutprobenentnahme)	Ja ☐				
		Nein ☐				
		Vielleicht ☐				
9.	Fliegen- und Mückenbekämpfung			Ja, *(Wie oft/ In welchen Abstände?)*		Nein
			Butox	☐		☐
			Latroxin-Delta	☐		☐
			Bayofly	☐		☐
			Andere:	☐		☐
10.	Sonstiges					

Legende: W= Wallach, H= Hengst,F= Stutfohlen, M=Hengstfohlen

Sollten BTV-Test Ergebnisse vorliegen, bitte ich Sie darum diese mir per Fax (0641-9938469) oder per Email (claudia.schulz@vetmed.uni-giessen.de) zuzusenden.-Vielen Dank!
Mit freundlichen Grüßen,
Claudia Schulz.

Appendix 6

Institut für Parasitologie, Justus-Liebig-Universität Gießen, Rudolf-Buchheim-Straße 2, 35392 Gießen,
Tel: 0641-9938478, Fax: 0641-9938469

Blutproben von Lamas und Alpakas
(Veterinärmed. Dissertation Claudia Schulz)

0

Betriebs-Nummer:
(nicht ausfüllen)

Datum: _____

Betriebsname: _____

Besitzer: _____

Adresse: _____

Tieranzahl: insgesamt: _____ genommene Blutproben: insges: _____

St.: M: Jährl: Fohlen:

Lfd.-Nr.	Tier-Name/ Chipnr.	Tierart		Geburts-datum	Geschlecht				Verwandt-schaft		Herkunft Woher & Wann?			Bemerkung (z.B. Erkrankungen, Auffälligkeiten, Herkunft,...)
		Alpaka(x)	Lama(x)	(Mo/J)	Stute	Männlich >2 J	Jährling (1-2J)	Fohlen	Mutter von	Fohlen von	Eigene Zucht	Fremd-betrieb	Ausland	
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														

Legende:

(x)	in die Spalte „Alpaka“ bzw. „Lama“ entsprechend ein Kreuz eintragen
Männlich	Hengste und Wallache >2 Jahre
Fohlen	"bei Fuß" bei der Mutter
Jährling	1-2 Jahre alte Stuten und Hengste
Stute	(Zucht-)stute >2 Jahre

LIST OF TABLES

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XII. REFERENCES

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XIII. ABBREVIATIONS

ADNS	Animal Disease Notification System
AGID	Agar gel immunodiffusion
AHSV	*African horse sickness virus*
BHK	Baby hamster kidney
BMELV	Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz
BT	Bluetongue
BTV	*Bluetongue virus*
C.	*Culicoides*
s.s.	*sensu stricto* (species)
s.l.	*sensu lato* (complex)
CLP	Core-like particle
CTL	Cytotoxic T-lymphocyte/ -cell
C_q	Quantification cycle
Cria	Offspring/calve of South American camelids
DEFRA	Department for Environment, Food and Rural Affairs
DIC	Disseminated intravascular coagulation
DIVA	Differentiating infected from vaccinated animals
(r)DNA	(Ribosomal) deoxyribonucleic acid
dpi	Days post-infection
EC	European Commission
ECs	Endothelial cells
ECE	Embryonated chicken eggs
EFSA	European Food Safety Authority
EIP	Extrinsic incubation period
EHDV	*Epizootic hemorrhagic disease virus*
(c)ELISA	(Competitive) Enzyme-linked immunosorbent assay
DR ELISA	Double recognition/double antigen ELISA
EMA	European Medicines Agency
EU	European Union
FLI	Friedrich-Loeffler-Institut
HCAb	Heavy-chain antibodies

IAH	Institute for Animal Health
IFN(-α)	Interferon(-alpha)
$IFNAR^{-/-}$	Interferon alpha/beta receptor deficient mice
Ig(G)	Immunoglobulin (G)
i.m.	Intramuscular
KC-cells	Cultured cells derived from *Culicoides sonorensis* embryos
MLV	Modified-live virus
NAbs	Neutralising antibodies
NS	Non-structural protein
OIE	World Organisation for Animal Health (Office International des Epizooties)
OWC	Old World camelids
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
p.n.	Post natum
p.v.	Post vaccination
RBC	Red blood cells
(ds)RNA	(Double-stranded) ribonucleic acid
mRNA	Messenger RNA
ssRNA	Single-stranded RNA
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
S(2)	Genome segment (2)
SAC	South American camelids
SBV	Schmallenberg virus
s.c.	Subcutaneous
SNT	Serum neutralisation test
spp.	Species pluralis, several species
TOV	Toggenburg orbivirus
TLR	Toll-like receptor
VIB	Virus inclusion body
ViP	Viral inclusion body matrix protein
VLP	Virus-like particle
VP	Viral structural protein

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