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Application of stochastic models to assess the probability of introduction and persistence of bluetongue in an area

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Jordi Casal i Fàbrega, catedràtic del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària i amb adscripció al Centre de Recerca en Sanitat Animal (CReSA) de la Universitat Autònoma de Barcelona,

FAIG CONSTAR,

Que el treball "Application of stochastic models to assess the probability of introduction and persistence of bluetongue in an area", presentat per Sebastián Napp Avelli per a l'obtenció del títol de Doctor, ha estat realitzat en el Centre de Recerca en Sanitat Animal (CReSA) sota la meva direcció.

Per a que consti, signo la present

Jordi Casal i Fàbrega

Bellaterra, 14 d'octubre de 2010

"Essentially, all models are wrong, but some are useful"

George E. Pelham Box
Statistician

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ABBREVIATIONS

Abbreviations	Meaning
AHSV	African horse sickness virus
BT	Bluetongue
BTV	Bluetongue virus
DAFF	Department of Agriculture, Fisheries and Forestry (Australia)
DEFRA	Department for Environment, Food and Rural Affairs (UK)
EEV	Equine encephalosis virus
EFSA	European Food Safety Authority
EHDV	Epizootic haemorrhagic disease virus
EIP	Extrinsic Incubation Period
ELISA	Enzyme-Linked ImmunoSorbent Assay
IETS	International Embryo Transfer Society
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PLVA	Period of low vector activity
SCC	Semen collection centre (bovines)
Se	Sensitivity
TITD	Time from *Culicoides* infection to its death
TITV	Time from Infection to Viraemia
TNBM	Time to the Next Blood Meal
TOV	Toggenburg Orbivirus
TTCI	Time To Culicoides Infection
VFP	Vector-free period
WTO	World Trade Organisation

1. INTRODUCTION

1.1. BLUETONGUE

1.1.1. ETIOLOGY

Bluetongue (BT) is a disease caused by bluetongue virus (BTV), a member of the genus *Orbivirus*, family *Reoviridae* (Mertens et al. 2004).

The family *Reoviridae* contains 15 genera of multi-segmented double-stranded (ds) RNA viruses, some of which are pathogens of a wide range of mammals (including humans), reptiles, fish, insects, crustaceans, plants and fungi, and many of them are of economic, veterinary or medical importance (Mertens et al. 2004; Mertens et al. 2008).

The members of the genus *Orbivirus* have a ten-segmented dsRNA genome within an icosahedral protein capsid (Mertens et al. 2004). The genus *Orbivirus* is the largest of the genera within the family *Reoviridae,* containing 22 distinct virus species and 10 further 'unassigned', not yet fully characterized viruses (Mertens et al. 2008). Besides BTV, the genus *Orbivirus* includes other viruses responsible of severe and economically important diseases of domestic and wild animals such as the African horse sickness virus (AHSV), the epizootic haemorrhagic disease virus (EHDV) and the equine encephalosis virus (EEV) (Mertens et al. 2004).

The bluetongue virus has 24 serotypes, distinguished on the basis of the antigenic profile of its major outer capsid protein VP2 (Schwartz-Cornil et al. 2008). However, in 2008, a novel bluetongue virus (BTV) termed Toggenburg orbivirus (TOV) was detected in goats from Switzerland (Hofmann et al. 2008).

1.1.2. CLINICAL SIGNS

Bluetongue (BT) affects mainly sheep and some species of wild ruminants, while BTV infection of cattle, goats and most wild ruminant species is typically asymptomatic or subclinical (MacLachlan 1994; Verwoerd & Erasmus 2004). The clinical signs of BTV infection are also highly variable even in susceptible species such as sheep, reflecting inherent differences in the susceptibility of different sheep breeds, as well as of individual animals. Besides, it seems that BTV-strains differ in their virulence for sheep (Sellers 1984).

The signs of BT in sheep are the result of virus-mediated vascular injury that produces hyperaemia and vascular congestion, oedema, haemorrhage and tissue infarction. Thus, sheep with acute BT may have fever, anorexia, respiratory distress, excessive salivation, serous to bloody nasal and ocular discharge that becomes mucopurulent so that crusty exudates accumulate around the nostrils, haemorrhages in the oral and nasal cavities, oral erosions and ulcers, lameness, hyperaemia and haemorrhage of the coronary band, oedema of the head and neck and congestion and focal haemorrhages in the conjunctiva and skin (Mertens et al. 2008). The swollen and cyanotic tongue that gives the disease its name is uncommon. Mortality rates vary from 0% in mild outbreaks to 30% or even higher in outbreaks caused by virulent strains of BTV in highly susceptible breeds of sheep.

Cattle exposed to BT virus under natural field conditions occasionally develop clinical signs of disease similar to those in BT-infected sheep, but in most instances the disease is unapparent (Luedke et al. 1970). Differences in clinical presentation between cattle and sheep seem to be related to differences in the susceptibility of endothelial cells from cattle and sheep to BTV infection (DeMaula et al. 2001; DeMaula et al. 2002a; DeMaula et al. 2002b).

1.1.3. EPIDEMIOLOGY

1.1.3.1. BLUETONGUE DISTRIBUTION

BTV is transmitted between its vertebrate hosts almost exclusively by the bites of haematophagus midges of the genus *Culicoides*. Consequently, its world distribution is limited to geographical areas where competent vector species of *Culicoides* are present, and its transmission to those periods of the year when climatic conditions are (1) favourable for adult vector activity and (2) temperatures warm enough to allow first the virus replication within the vector, and then transmission to a susceptible host (Mertens et al. 2008).

Therefore, the disease was considered to be confined to tropical and subtropical areas of the world, between latitudes 35°S and 40°N, where the known competent vector species occurred (Purse et al. 2005) (figure 1).

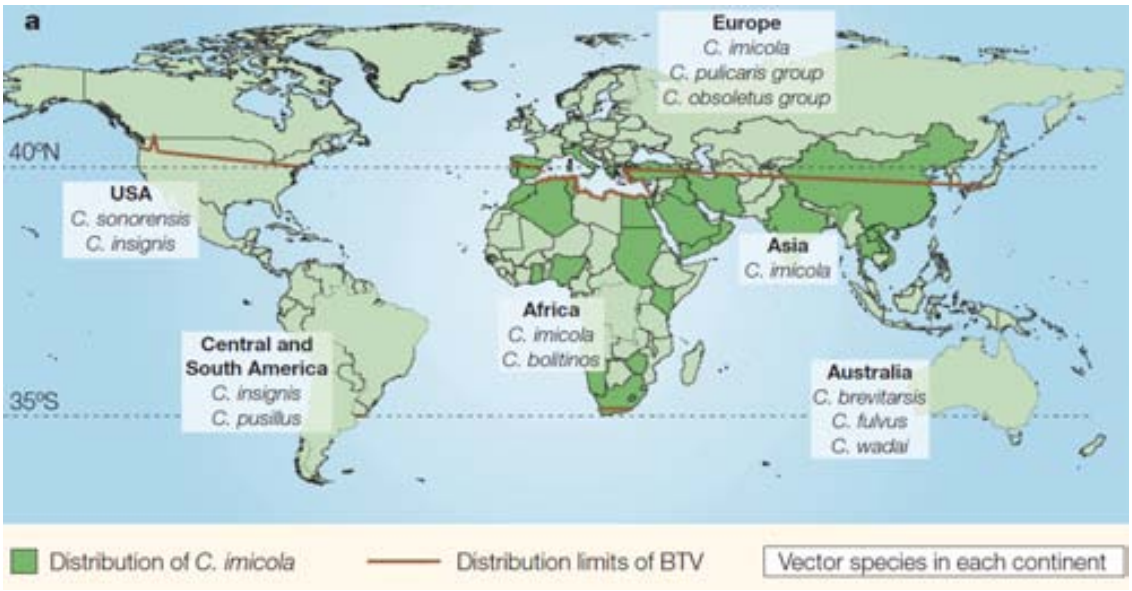

Figure 1: Distribution of BTV and *Culicoides* vectors (from Purse et al. 2005)

Bluetongue in Europe

Before 1998

Before 1998, the distribution of BTV in Europe coincided with the known distribution of the Afro-Asiatic species *C. imicola*, and besides Cyprus, where BT occurred regularly, only two epidemics occurred. The first epidemic affected the South of Spain and Portugal between 1956 and 1960, and was caused by BTV-10, which entered the Iberian Peninsula from Morocco (Campano Lopez & Sanchez Botija 1958; Vassalos 1980). The second epidemic affected the Greek Islands of Rhodes and Lesbos between 1979 and 1980, and was caused by BTV-4 (Vassalos 1980).

This limited number of epidemics led European countries to believe that the risk of BT epidemics was low (Carpenter et al. 2009a).

Between 1998 and 2005

In October 1998, BTV-9 affected several Greek islands close to the Anatolian Turkish coast. In the following years up to 2004, BTV-9 spread northward (into western Turkey, Bulgaria, Kosovo, Albania, Bosnia and Herzegovina, Macedonia, Serbia, Montenegro, and Croatia) and westward (into mainland Greece, Italy, Sicily, Sardinia and Corsica) (Purse et al. 2005). Three other serotypes, BTV-1, BTV-4 and BTV-16, also entered Europe from the east (through Greece) and then spread westwards.

The incursions of BTV serotypes 1, 4, 9 and 16 into the eastern part of the Mediterranean Basin clearly originated from the east of Europe. BTV serotypes 4, 9 and 16 are known to have circulated in Turkey, Syria, Jordan and/or Israel (Mertens et al. 2008). On the other hand, serotypes 1 had not previously been reported from these areas, but genetic studies demonstrated that BTV-1 European isolates were closely linked to isolates of the serotype from India (Maan et al. 2004). Therefore it is likely that BTV-1 was also present in Turkey and/or the Middle East but was not detected (Mertens et al. 2008).

Besides, in 2000, a separate incursion of BTV-2 also occurred, spreading from Tunisia and/or Algeria into Sardinia, Sicily, mainland Italy, Corsica and the Balearic islands (Purse et al. 2005).

In 2004, BTV-4 was detected in Morocco from where it spread to southwestern Spain and southern Portugal (Purse et al. 2005).

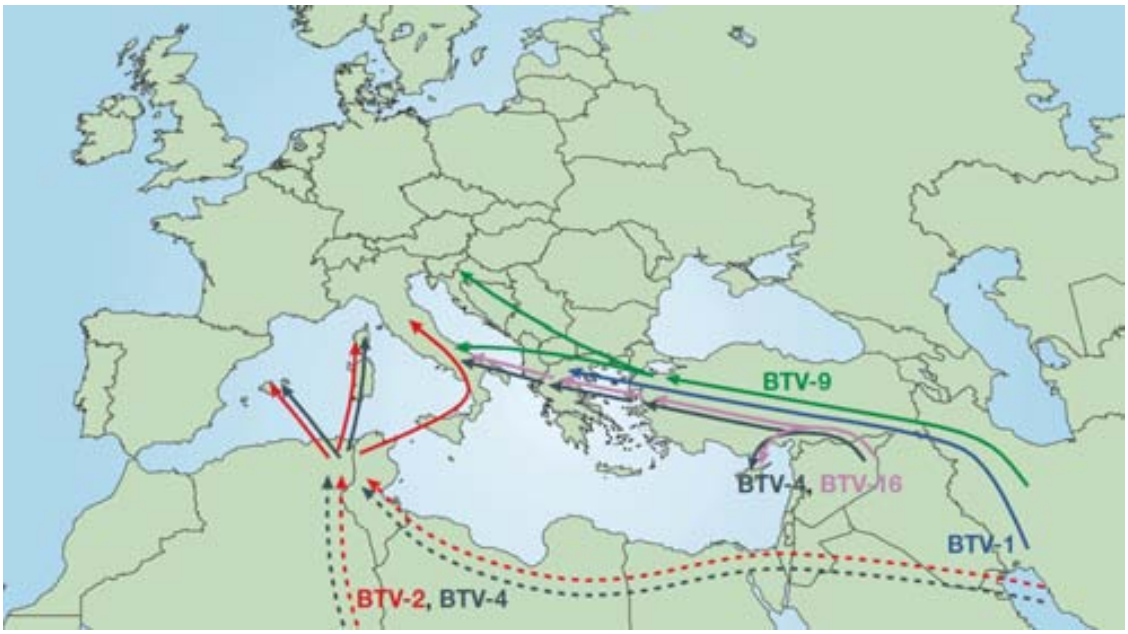

Figure 2: Routes of origin of BTV serotypes introduced into Europe between 1998 and 2005 (from Purse et al. 2005)

There are several features that indicate that a substantial change in the epidemiology of BT in Europe since 1998: first, the expansion of BT to areas not previously affected; second, the almost simultaneous incursion of different BTV serotypes; third, the persistence of different BTV serotypes for several years; fourth, the northern extension of the range limit of the traditional vector *C. imicola*; and fifth, the implication of Palearctic *Culicoides* species in BTV transmission (Purse et al. 2005).

From 2006 to 2010

In August 2006, an outbreak of BTV-8 was detected near Maastricht (the Netherlands), from where it spread to most of the country and to virtually the whole of Belgium, North-West Germany, Luxembourg and the northern borders of France (Wilson & Mellor 2008).

By the end of 2006, as a consequence of the cooler weather, the number of BTV-8 outbreaks in northern Europe decreased and finally ended. By the end of 2006, over 2000 outbreaks had been declared (EFSA 2007). There was no evidence of BTV transmission in northern Europe during the first few months of 2007, but in May 2007, a sentinel bovine seroconverted in NW Germany, which was the first evidence that BTV-8 had successfully overwintered in northern Europe (Hoffmann et al. 2008). The virus subsequently resurfaced in all countries affected in 2006, with new cases occurring for the first time in Denmark, Switzerland, the Czech Republic and the UK (Wilson & Mellor 2009). The 2007 epidemic was far more devastating than that of 2006, and by the end of 2007 nearly 60.000 holdings had been infected. BTV-8 overwintered again, but the number of outbreaks in 2008 was significantly reduced as a consequence of the

establishment of vaccination programmes in the affected European countries (Wilson & Mellor 2009). Besides, the first case of BTV-8 was detected in Cantabria, northern Spain (Wilson & Mellor 2009).

In July 2007, a new serotype (BTV-1), which was circulating in Morocco reached southern Spain (Allepuz et al. 2010), from where it spread to other Spanish regions, Portugal and finally northern France.

Two other serotypes, BTV-6 and BTV-11, both closely related to South-African vaccine strains were detected in the Netherlands in October 2008 and Belgium in January 2009 (Batten et al. 2010; De Clercq et al. 2009).

Finally, in 2008 BTV surveillance in Switzerland detected the presence of a novel BT-like virus provisionally termed Toggenburg Orbivirus (TOV), genetically distinct from any other BTV, which resulted in subclinical low viraemia infections in goats (Hofmann et al. 2008).

1.1.3.2. MECHANISMS OF BTV INTRODUCTION INTO A FREE AREA

Introduction of BTV into a free area may occur in four ways. The first is through movement of infected animals (domestic and wild ruminants) or animal germplasm (semen, embryos). The second is by infected vector *Culicoides* carried by various living (plants, animals) or inanimate (airplanes, ships) means. The third is through the active flight of infected vector *Culicoides* and the fourth is through passive flight of infected vector *Culicoides* by the wind (Saegerman et al. 2008).

a) Movement of infected animals (domestic and wild ruminants) or animal germplasm (semen, embryos).

Movement of infected domestic ruminants

The movement of infected animals has often been ruled out as the cause of BTV introduction into free areas (Miranda et al. 2003; Calistri et al. 2004; Mintiens et al. 2008a). On the other hand, the introductions in Europe of BTV serotypes 1, 9 and 16 were probably attributable to animal movements along the “Eurasian ruminant street” (an area with high densities of ruminants stretching from India and Pakistan through Afghanistan, Turkey, Iraq and Iran to the southeast of Europe)(Wilson & Mellor 2009), which is also believed to contribute to the spread of other livestock diseases such as foot-and-mouth disease (Slingenbergh et al. 2004). Besides, the route of entry of BTV-2 into Algeria in 2000 and later to Italy, is uncertain but it is likely that BTV could have followed a similar route to that of foot and mouth disease virus, which

was thought to have entered into Algeria in 1999 via cattle smuggled from the Ivory Coast and Guinea (Mertens et al. 2008).

Besides, it is known that movement of infected animals plays an important in the further spread of the disease as demonstrated with BTV-4 in Spain in 2004 (Mertens et al. 2008).

Movement of infected wild ruminants

The circulation of BTV has been described in a wide range of wild ruminant species in many countries from different continents (García et al. 2009). In a study carried out in Southern Spain, seropositive wild ruminants were detected in areas where BTV outbreaks had not been detected in domestic livestock. The fact that BTV circulated in wild ruminants in areas where bluetongue was not detected in domestic livestock suggests that wild ruminants may play a role in the epidemiology of BTV in certain areas. The importance of this role is likely to be dependent upon their population levels and their proximity to domestic livestock. Translocations of wild ungulate species are common, and the risk of introducing pathogens into disease free areas by such movements should be also taken into account (Gortázar et al. 2006).

Import of infected semen

The assumption that bulls may shed BTV in their semen derived from the studies carried out in the 1970s (Luedke et al. 1977; Breckon et al. 1980), and that led to constraints in the international trade of semen. However, further attempts were not able to confirm this theory and this failure was attributed to the intermittent excretion of BTV in semen. The possibility of virus shedding seemed to be related to the type of virus ("wild type" *vs.* laboratory-adapted) and to the age of the animals (Kirkland & Hawkes 2004): BTV was often detected in semen of old bulls infected with laboratory adapted viruses, and in semen of some old bulls infected with "wild" strains, although it was believed that the virus was present in semen as a result of inflammation or because of the presence of blood in semen. The uncertainties regarding the epidemiology of BTV were used to justify protectionist trade barriers imposed by some BTV-free countries with severe economic consequences (MacLachlan & Osburn 2006). However, the presence of live BTV was recently confirmed in 54% of the semen samples from bulls naturally infected with BTV-8, by a combination of PCR and virus isolation (Vanbinst et al. 2010). The fact that virulent wild-type BTV-8 is shed easily in semen indicates that there are important differences in the probability of BTV shedding in semen depending on the serotype.

Import of infected embryos

With regard to the possibility that BTV might be transmitted via in vivo-derived embryos, there have been several experiments which convincingly showed, at least for embryos washed

according to the International Embryo Transfer Society (IETS) protocols, that the virus is not so transmitted (Wrathall et al. 2006).

b) Infected vector *Culicoides* carried by various living (plants, animals) or inanimate (airplanes, ships) means.

Infected *Culicoides* carried by plants

Whereas many insect species are associated with plants, breeding or feeding on them, and consequently may be transported with them, this is not known to be the case for the haematophagous *Culicoides* vectors of BTV. There are no references reporting *Culicoides* being found in imported flowers or plants. However, flowers which are exported from Africa into Europe are packed at night under bright artificial light. If a flower-packing station was near a farm it was theoretically possible that *Culicoides* were attracted from the farm into the flower station and were included in the polythene packing round the flowers. It is known that when the packing is removed in Europe 'clouds of insects' are released (R. Meiswinkel, personal communication).

Infected *Culicoides* carried by (non-ruminant) animals

Adult *Culicoides* associate much more closely with their mammalian hosts than with plant species (Mintiens et al. 2008a). Important *Culicoides* species such as *C. imicola*, *C. brevitarsis* and *C. obsoletus* are opportunistic feeders biting a wide range of livestock including sheep, cattle, goats, horses and pigs (EFSA 2007). Therefore, infected *Culicoides* may potentially be introduced along with non-ruminants animals.

Infected *Culicoides* carried by airplanes or ships

Air, sea and land transport networks continue to expand and pathogens and their vectors can now move further, faster and in greater numbers than ever before (Tatem et al. 2006). One of the important consequences of global transport network expansion is vector-borne pathogen importation.

Even though the introduction of insects and their pathogens via ships and aircraft is well known (Gratz et al. 2000; Lounibos 2002), *Culicoides* because of its small size, fragile nature and specialist taxonomy, have been mainly overlooked and therefore information is scarce (Carpenter et al. 2009a). Nie and collaborators (Nie et al. 2005) found *Culicoides* in 9 out of the 70 ships inspected at Qinhuangdao port, China. Reye (Reye 1964) reported the probably spread of *Culicoides* by aircraft from Fiji to the Society Islands. In fact, some countries, concerned with this risk have implemented surveillance for *Culicoides* at border controls, e.g. the Australian Quarantine and Inspection Service (Carpenter et al. 2009a).

c) Active flight of infected vector *Culicoides*

In general, the active dispersal of *Culicoides* by flight is usually short and most species disperse only a few hundred meters from their breeding sites or at most 2–3 km/day (Mellor et al. 2000). It seems that active flight of *Culicoides* is more related to the local spread than to long distance spread (Gerbier et al. 2008; Saegerman et al. 2008).

d) Passive flight of infected vector *Culicoides* by the wind

Thanks to their small size, *Culicoides* are likely to be transported on the wind as aerial plankton (Wittmann & Baylis 2000). Given some conditions: winds at speeds of 10–40 km/h, at heights up to 1.5 km and at temperatures between 12 and 35°C, *Culicoides* have been postulated to be transported over distances up to 700 km over water (Ducheyne et al. 2007) and 150 Km over land (Hendrickx et al. 2008).

The passive transportation of infected *Culicoides* on the wind has been hypothesized as the mechanisms of BTV introduction into free areas on many occasions (EFSA 2007): BTV-4 into Cyprus from Syria and Turkey in 1977 (Sellers et al. 1979); BTV-2 into Sardinia and Sicily from Algeria (Calistri et al. 2004) and then from Sardinia into the Balearic islands (Alba et al. 2004) in 2000; BTV-4 into Southern Spain from Morocco in 2004 (Mertens et al. 2008); or BTV-8 into the UK from Belgium in 2007 (Gloster et al. 2008).

1.1.3.3. MECHANISMS OF BTV PERSISTENCE DURING WINTER (OVERWINTERING)

Low temperatures in winter reduce the activity of vectors and BTV replication within them, and therefore BTV transmission is apparently interrupted. However, after winter, transmission is often resumed, a process which is known as overwintering (Wilson et al. 2008). A large number of mechanisms to explain BTV overwintering have been proposed.

Most *Culicoides* at northern latitudes survive the winter as larvae, and therefore the most logical explanation for overwintering was thought to be the vertical (transovarial) transmission of the virus from infected adult vectors to offspring (Wilson et al. 2008). Even though viral RNA was detected in larvae by PCR (White et al. 2005) BTV could not be isolated. The efficiency of transovarial transmission may vary dramatically with the species of insect vector and virus. Although the reasons are unknown, this type of transmission seems to be restricted to mosquitoes and phlebotomid sandflies, as none of the more than 50 arboviruses isolated from *Culicoides* species worldwide are known or suspected to be transovarially transmitted (Mellor 2000).

Entomological surveillance systems in Northern Europe have demonstrated that small populations of *Culicoides* remain active during winter (Wittmann et al. 2002; Losson et al.

2007), and therefore year-round presence of adult infected *Culicoides* was considered as the most likely explanation for sustenance of the transmission cycle (EFSA 2007).

Persistence of BTV in the ruminant population may also occur by transmission between ruminants during sexual intercourse. Infected bulls were reported to shed BTV in semen, although it seemed to be restricted to old bulls and laboratory adapted viruses as there was no published report of isolation of BTV from semen of naturally infected bulls (Kirkland et al. 2004). However, in a recent study (Vanbinst et al. 2010), were able to isolate BTV-8 from a significant proportion of semen samples of viraemic bulls.

Transmission of BTV-8 by direct contact, probably through ingestion of infected placentas, has also been reported (Menzies et al. 2008). Vertical (transplacental) transmission of BTV has been described in both cattle and sheep, but was thought to be exclusively associated to cell-attenuated virus strains (Backx et al. 2009). Nevertheless, in the case of BTV-8, transplacental transmission has been demonstrated both in the field (De Clercq et al. 2008; Menzies et al. 2008; Darpel et al. 2009; Santman-Berends et al. 2010) and experimentally (Backx et al. 2009), although, at least in naturally-infected sheep, its contribution to overwintering appears to be limited (Saegerman et al. 2010).

BTV may also persist in the ruminant population during the winter, through a prolonged viraemia in some individuals. Infectious BTV can be isolated from the blood of cattle for much longer than from sheep and goats, and although the vast majority of infections in cattle endure for less than 60 days, a fraction may last for much longer (Wilson et al. 2008). Such infections could permit the virus to persist for months without infecting new hosts, and thereby survive short periods of vector absence.

Bouwknegt and collaborators (Bouwknegt et al. 2010) demonstrated that BTV-8 can survive for at least 21 days in ixodid ticks and up to 26 days in the soft tick *Ornithodoros savignyi*. BTV can pass the gut barrier to the salivary glands, ovaries and testes, allowing transmission trans-stadially and intra-stadially in male *Ixodes* ticks. Besides, in *O. savignyi* can be transmitted transovarially. Although, further studies to investigate transmission from infected ticks to domestic livestock are required, this route of transmission could provide a potential overwintering mechanism for bluetongue virus.

Takamatsu and collaborators (Takamatsu et al. 2003) showed that BTV can persistently infect ovine $\gamma\delta$ T-cells *in vitro*, a process that if occurred also during infection and viraemia in the mammalian hosts, would provide a mechanism for virus persistence. However, given their failure to recover live virus from their persistently infected sheep by naive vector insect bite, as well as the failure to isolate viable BTV from the blood or skin samples that were processed

normally, some authors (White & Mecham 2004) concluded that Takamatsu's results could have been an artifact.

Finally, BTV might be maintained in an as yet unknown reservoir host (Wilson et al. 2008). Given that BTV-8 infection of wild cervids was unapparent, that the seroprevalence in red deer was high, and that spleen samples from dead red deer found during winter were positive by PCR, Linden and collaborators speculated that red deer may act as BTV reservoirs (Linden et al. 2010).

1.2. RISK ASSESSMENT

1.2.1. IMPORT RISK ANALYSIS

1.2.1.1. INTRODUCTION

The importations of animals or animal products pose a risk to the importing country as a consequence of the possibility of introduction of a disease.

To protect against such risks, the Agreement on the Application of Sanitary and Phytosanitary Measures (known as the SPS agreement) of the World Trade Organisation (WTO) allows WTO Member Countries two options (OIE 2004a):

a) Base their sanitary measures on international standards such as the World Organisation for Animal Health (OIE) *Terrestrial Animal Health Code.*

b) In the absence of relevant standards or when Members choose to adopt a higher level of protection, science-based risk analysis is essential to determine whether importation of a particular commodity implies a significant risk to animal or human health and if so, what sanitary measures may be applied to reduce that risk to an acceptable level.

Risk analysis is a tool intended to provide decision-makers with an objective, repeatable and documented assessment of the risks posed by a particular course of action. The main aim of import risk analysis is to provide importing countries with an impartial and defensible method of assessing the risk associated with the importation of animals or animal products.

1.2.1.2. OIE RISK ANALYSIS FRAMEWORK

Although some principles of the risk analysis methodology had been used for a long time, it was not until the 1990s that the need for a standardized method was acknowledged. With the aim of providing an international reference text on import risk analysis, the OIE developed the Handbook on Import Risk Analysis for Animals and Animal Products.

The OIE methodology is based on the system developed by Covello and Merkhofer, in which the risk analysis process is composed of 4 separate steps (figure 3):

1- Hazard identification

2- Risk assessment

3- Risk management

4- Risk communication

Figure 3: Structure of the OIE risk analysis process

1- Hazard identification

It involves assessing the pathogenic agents which could potentially produce adverse consequences (hazards) associated with the importation of a commodity.

Depending on the category of the commodity, some pathogenic agents may not need to be further considered. The methods of production, manufacturing or processing may also result in the exclusion of some pathogens (OIE 2004a).

2- Risk Assessment

It involves the evaluation of the likelihood and the (biological and economic) consequences of entry, establishment and spread of a hazard within an importing country.

A risk assessment consists of 4 inter-related steps:

a) Release assessment

It consists of describing the biological pathway(s) necessary for an importing commodity to 'release' (i.e., introduce) each potential hazard into the importing country, and estimating the probability of that complete process occurring, either qualitatively (in words) or quantitatively (as a numerical estimate).

b) Exposure assessment

It consists of describing the biological pathway(s) necessary for the exposure of animals or humans in the importing country to the hazards (pathogenic agents) previously released, and estimating (qualitatively or quantitatively) the probability of the exposure(s) occurring.

c) Consequence assessment

The consequence assessment describes the potential consequences of a given exposure and estimates the probability of them occurring, either qualitatively or quantitatively.

d) Risk estimation

It consists of integrating the results from the release, exposure, and consequence assessments to produce overall measures of risks associated with the hazards previously identified.

3- Risk Management

It is the process of deciding upon and implementing sanitary measures to effectively manage the risks posed by the hazard(s). The objective is to ensure that a balance is achieved between a country's desire to minimize the probability of importation of a hazard and their consequences and its desire to import commodities and fulfil its obligations under international trade agreements.

4- Risk Communication

Is the process by which information and opinions regarding hazards and risks are gathered from potentially affected and interested parties during a risk analysis, and by which the results of the risk assessment and proposed risk management measures are communicated to the decision-makers and interested parties in the importing and exporting countries. It is a multidimensional and iterative process and should ideally begin at the start of the risk analysis process and continue throughout.

1.2.2. TYPES OF RISK ASSESSMENT

No single risk assessment method is applicable in all cases, and different techniques are available for different circumstances. Risk assessment should be able to accommodate the variety of animal commodities, the multiple hazards that may be identified with an importation and the specificity of each disease, detection and surveillance systems, exposure scenarios and types and amounts of data and information.

There are 2 main types of risk assessment: qualitative risk assessment and quantitative risk assessment.

Qualitative risk assessment

It is an assessment where the likelihood of the outcome or the magnitude of the consequences are expressed in qualitative terms such as ‘high’, ‘medium’, ‘low’ or ‘negligible’. It is usually used as an initial approach, before carrying out a quantitative risk assessment, or when numerical data is not available.

It is employed by governments in some countries such as the U.K. (DEFRA 2010) or Australia (DAFF 2010), to assess whether the emergence of a disease anywhere in the world, poses a risk to the country.

Quantitative risk assessment

When numerical data are available, a quantitative risk assessment may be undertaken. It is an assessment where the likelihood of the outcome or the magnitude of the consequences are expressed numerically.

There are 2 types of quantitative risk assessment:

a) Deterministic (point estimate) risk assessment

The inputs (and therefore the outputs) are expressed as single values. They may represent the 'expected value', 'the mean value', and in some occasions, the value which corresponds to the 'worst-case scenario'.

b) Probabilistic (stochastic) risk assessment

Developments in computer software have enabled the use of probability distributions to describe inputs which are uncertain and/or variable[‡] in nature. This results in a stochastic model in which the output(s) is also described by a probability distribution.

Sampling values from the input probability distributions are usually undertaken by either Monte Carlo or Latin Hypercube sampling. The Monte Carlo method is based on simple random sampling from a probability distribution, while Latin Hypercube sampling involves stratified sampling (OIE 2004b).

‡Uncertainty represents the lack of knowledge about a given parameter and is reducible through the collection of additional information, while variability is the effect of chance and cannot be reduced through the collection of further data (Vose, 2000)

2. OBJECTIVES

The objective of **study I** was to assess the probability of BTV overwintering by horizontal transmission by persistence of the virus in adult vectors, in ruminants (through prolonged viraemia) or in a combination of both, by means of a stochastic risk assessment model. Besides, the model allowed assessing the role that the few *Culicoides* present during the period of low vector activity (*PLVA*) and those which live inside buildings (endophilic *Culicoides*) play on the probability of overwintering. The model was applied to a real scenario: overwintering in Germany between 2006 and 2007.

The objective of **study II** was to assess, by means of a stochastic risk assessment model, the probability of development of a BTV outbreak as a consequence of the introduction of infected *Culicoides* via transport and trade networks. The model was applied to calculate the risk of a BTV-8 epidemic in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries (Germany, Belgium, the Netherlands, Luxemburg, France, the Czech Republic, Denmark and the UK), regardless of the mechanism by which the midges were introduced.

The objectives of **study III** were to assess, in case of introduction of BTV into a bovine semen collection centre (SCC), both the risk of BTV transmission by semen and the risk reduction achieved by some of the preventive measures available. In order to do this, a stochastic risk assessment model was constructed. The model was applied to different scenarios, constructed according to: a) the type of diagnostic test and interval between the controls of donor bulls (either ELISA every 60 days or PCR every 28 days), b) the rate of BTV spread within the SCC (either low or high), and c) the timing of tests (either simultaneous or non-simultaneous). Besides, the effectiveness of testing the semen samples was also assessed.

3. STUDIES

3.1. STUDY I: Quantitative assessment of the probability of bluetongue virus overwintering by horizontal transmission: application to Germany

3.1.1. Introduction

Bluetongue (BT) is a non-contagious disease of ruminants, mainly sheep, caused by BTV, which belongs to the genus *Orbivirus* within the family Reoviridae. It is transmitted between hosts almost exclusively through the bites of the females of the *Culicoides* biting midge. BT is an OIE reportable disease and is of considerable socioeconomic concern and of major importance in the international trade of animals and animal products (Saegerman et al. 2008). Before 1998, BT was considered an exotic disease in Europe with just a few sporadic incursions, but between 1998 and 2005, different BTV strains affected several countries in the Mediterranean basin. In August 2006, BTV-8 was identified in the Netherlands, from where the disease spread to neighbouring countries. After a short winter break, BTV reappeared in 2007 causing a devastating epidemic (Wilson & Mellor 2009). Transmission of BTV is apparently interrupted during winter as a consequence of the low temperatures, which reduce the activity of vectors and BTV replication within them. However, once winter is finished, transmission often restarts (Wilson et al. 2008). Several different mechanisms have been proposed to explain BTV overwintering.

Most *Culicoides* at northern latitudes survive the winter as larvae, and therefore the most logical explanation for overwintering was thought to be the vertical (transovarial) transmission of the virus from infected adult vectors to offspring (Wilson et al. 2008). However, even though White and collaborators were able to detect viral RNA in larvae by PCR (White et al. 2005), they were not able to isolate the virus. Persistence of BTV in the ruminant population may also occur by transmission between ruminants during sexual intercourse. Infected bulls may shed BTV in semen, but it seemed to be restricted to old bulls and laboratory adapted viruses as there was no published report of isolation of BTV from semen of naturally infected bulls (Kirkland et al. 2004). However, in a recent study (Vanbinst et al. 2010), the presence of BTV-8 in semen of viraemic bulls was detected by PCR and virus isolation.

Transmission of BTV-8 by direct contact, probably through ingestion of infected placentas, has also been reported (Menzies et al. 2008). Vertical (transplacental) transmission of BTV has been described in both cattle and sheep, but was thought to be exclusively associated to cell-attenuated virus strains (Backx et al. 2009). Nevertheless, in the case of BTV-8, transplacental transmission has been demonstrated both in the field (Menzies et al. 2008; De Clercq et al. 2008; Darpel et al. 2009; Santman-Berends et al. 2010) and experimentally (Backx et al. 2009), although, at least in naturally-infected sheep, its contribution to overwintering appears to be limited (Saegerman et al. 2010). Besides, several other mechanisms for overwintering, which are not yet sufficiently proven, have been proposed: a) unidentified reservoir hosts (Wilson et

al. 2008), b) alternative vectors such as ticks or biting flies (Wilson et al. 2008; Bouwknegt et al. 2010), or c) persistently infected ovine γδ T-cells (Takamatsu et al. 2003).

However, before investigating all these particular overwintering mechanisms, it should first be clear how likely (ordinary) horizontal transmission could be responsible. This paper deals with the assessment of the probability of bluetongue virus overwintering by horizontal transmission. BTV may persist in the ruminant population during the winter, through a prolonged viraemia in some individuals. Infectious BTV can be isolated from the blood of cattle for much longer than from sheep and goats, and although the vast majority of infections in cattle endure for less than 60 days, a fraction may last for much longer (Wilson et al. 2008). Such infections could permit the virus to persist for months without infecting new hosts, and thereby survive the periods of vector absence. Besides, entomological surveillance systems in Northern Europe have demonstrated that small populations of *Culicoides* remain active during winter (Losson et al. 2007; Zimmer et al. 2010), and therefore year-round presence of adult infected *Culicoides* was considered as the most likely explanation for sustenance of the transmission cycle (EFSA 2008). Nevertheless, BTV does not need to survive solely in either the host or the adult vector, but the mechanism for overwintering may be a combination of both. A *Culicoides* may infect the host before the end of the winter and the virus may reach the next season in the blood of infected ruminants (mainly cattle), when the conditions (presence of *Culicoides*) allow the re-emergence of disease.

The complete cessation of vector activity during winter, i.e. the vector free period (VFP), seems to be restricted to Afro-tropical species such as *C. imicola*, and only in specific areas of southern Europe. In other areas of Europe and with other *Culicoides* species, a period of total cessation of adult vector activity seems not occur. However, it is possible to identify periods of the year when the risk of transmission of BTV may be considered very low. This low transmission period (i.e. Period of Low Vector Activity; *PLVA*), will vary across Europe depending on the timing and duration of the local climate (EFSA 2008), and the biology of the vector species involved.

The assumption that *Culicoides* are purely exophilic (they will not enter or rest inside buildings) was attributed to the fact that most studies were performed in tropical areas or in the Mediterranean, on exophagic species like *C. imicola* (Baldet et al. 2008). However, studies in Northern Europe, have demonstrated that *Culicoides* are regularly found inside buildings (Baldet et al. 2008; Meiswinkel et al. 2008a; Meiswinkel et al. 2008b; Clausen et al. 2009) and that the endophagic behaviour appears to be driven primarily by external temperatures (Baldet et al. 2008). The ability of *Culicoides* to shelter from cold conditions inside farm

buildings could extend the period of active BTV transmission (Carpenter et al. 2009b), and that may have an impact on the probability of overwintering.

Therefore, the aim of the manuscript was to assess the probability of BTV overwintering by horizontal transmission by persistence of the virus in either adult vectors, ruminants (through prolonged viraemia) or a combination of both, by means of a stochastic risk assessment model. Besides, the model allowed assessing the role that the few *Culicoides* present during the *PLVA* and those which live inside buildings play on the probability of overwintering. The model was applied to a real scenario: overwintering in Germany between 2006 and 2007.

3.1.2. Materials and Methods

3.1.2.1. Model pathways

The model allowed the estimation of the probability of overwintering by different pathways (figure 1):

I- Overwintering by long term persistence in the adult vector.

II- Overwintering by long term persistence in the ruminant host.

III- Overwintering by persistence in the vector plus the ruminant host.

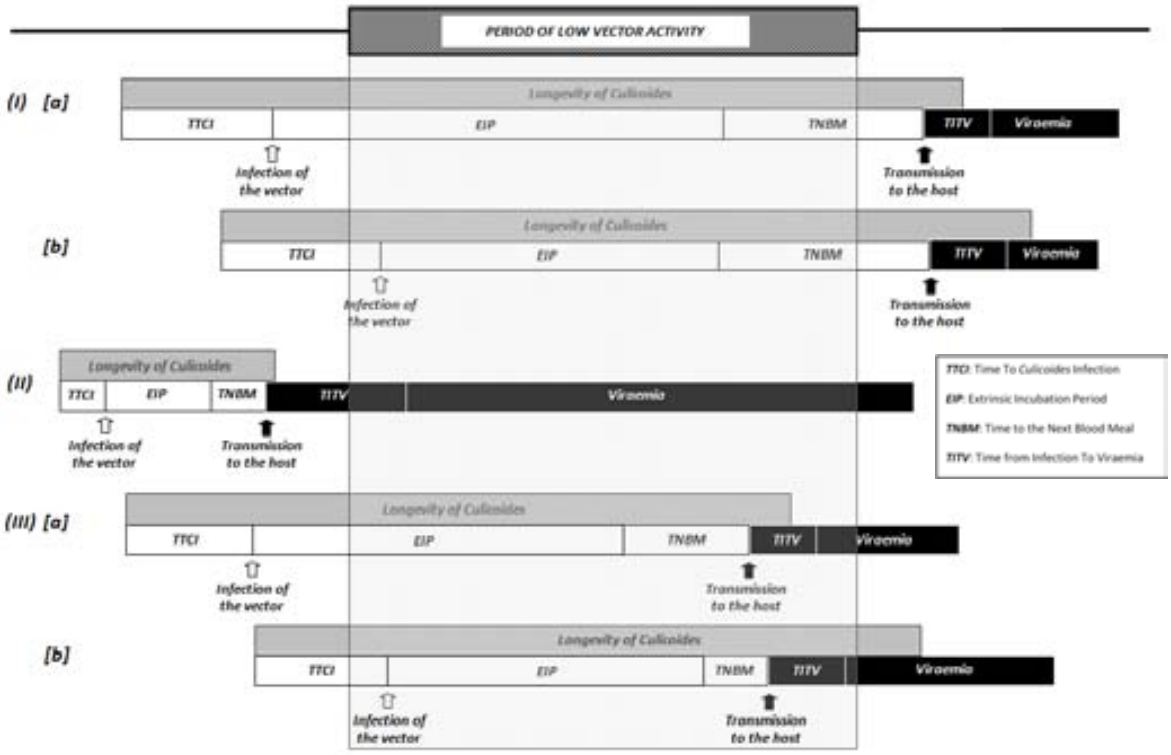

Figure 1: Pathways for overwintering considered in the model: (*I*) horizontal transmission in the insect vectors, (*II*) horizontal transmission in the ruminant hosts and (*III*) horizontal transmission in the insect vector plus the ruminant population. [a] represents infection of vectors before the *PLVA* and [b] infection of vectors during the *PLVA*. In pathways *Ia* and *IIIa*, the vectors need to have emerged before the *PLVA*, while in pathways *Ib* and *IIIb*, the vectors may have emerged before the *PLVA*, but also during the *PLVA*.

In order to be able to transmit BTV, the vector needs to: a) become infected (the number of days from the emergence of adult vectors to infection is called time to *Culicoides* infection (*TTCI*)), b) be able to survive the extrinsic incubation period (*EIP*) and the time to the next blood meal (*TNBM*), and, c) be able to effectively transmit BTV to a susceptible host. If the transmission to the host occurs beyond the *PLVA*, then overwintering was considered to have been achieved by persistence of BTV in the adult insect vectors (pathway *I*). If not, overwintering may still be achieved with the participation of the host. In this case, once the host becomes infected, there is a period until the animal becomes viraemic: time from infection to viraemia (*TIV*) and then a viraemic period. If the viraemic period goes beyond the end of the *PLVA*, then overwintering was considered to have been achieved by persistence of the virus in the adult vector plus the ruminant host (pathway *III*). If the host got infected before the start of the *PLVA* and the viraemic period went beyond the *PLVA*, then overwintering was considered to have been achieved by persistence of the virus in the ruminant hosts (pathway *II*).

In order to assess the role played by the small number of vectors present during the period of low vector activity, pathways *I & III* were further divided depending on whether the vectors were infected: [a] before the start of the *PLVA*, or, [b] during the *PLVA*.

Quantification of *Culicoides* population size is based on trapping, which samples only a proportion of the *Culicoides* population, although the exact size of this portion is not known (Meiswinkel et al. 2008a). Consequently, the probabilities for each pathway (*Ia, Ib, IIIa* & *IIIb*) had to be estimated per vector. However, the model does allow quantification of the relative importance of these four different pathways. For pathway *II*, the overall probability may be estimated because the ruminant population in an area or country is usually known.

In order to explore the effect of a proportion of *Culicoides* living inside buildings and therefore subjected to a milder temperature during the winter months, the model was run a) assuming exophilic behaviour exclusively and b) assuming a proportion of vectors had endophilic behaviour (this proportion given by the probability of endophily on that month).

The model allows the estimation of these probabilities taking into account the specific conditions in a given country or area: i) pattern of *Culicoides* activity throughout the year, ii) temperatures, iii) bluetongue incidence in both bovine and ovine in the previous season, and iv) cattle and sheep populations.

3.1.2.2. Risk assessment model

For overwintering to occur, a series of events (steps) have to take place (figure 2).

3.1.2.2.1. Probability of a *Culicoides* getting infected

Firstly, the probability of a *Culicoides* getting infected after a single blood meal was estimated as the product of: 1) the proportion of bites on cattle and sheep, 2) the probabilities of cattle and sheep being viraemic in month *i* (for *i*= November to April), and, 3) the proportion of bites on an infectious host that infect a midge.

Secondly, given a *Culicoides* which emerged on a given day, its longevity and the biting rate were calculated and used to estimate the number of blood meals the *Culicoides* had taken (*n*), which was then used to estimate the probability of infection after *n* blood meals.

3.1.2.2.2. Probability a *Culicoides* survives the *EIP* and the *TNBM*

Once the vector got infected, it needed to be able to survive the *EIP* (i.e. the time from the ingestion of the virus until it reaches the salivary glands) and the *TNBM*, so that BTV can be transmitted to a susceptible host.

3.1.2.2.3. Probability of effective transmission

Probability of effective transmission was estimated taking into account: 1) the proportion of bites on cattle and on sheep, 2) the proportion of cattle and sheep which are susceptible (not immune), and 3) the proportion of bites per infectious midge that infect a host.

3.1.2.2.4. Probability the viraemia goes beyond the end of the *PLVA* (for pathways *II* & *III*).

This probability was estimated taking into account: 1) the time from infection to viraemia, and 2) the duration of viraemia in cattle or sheep.

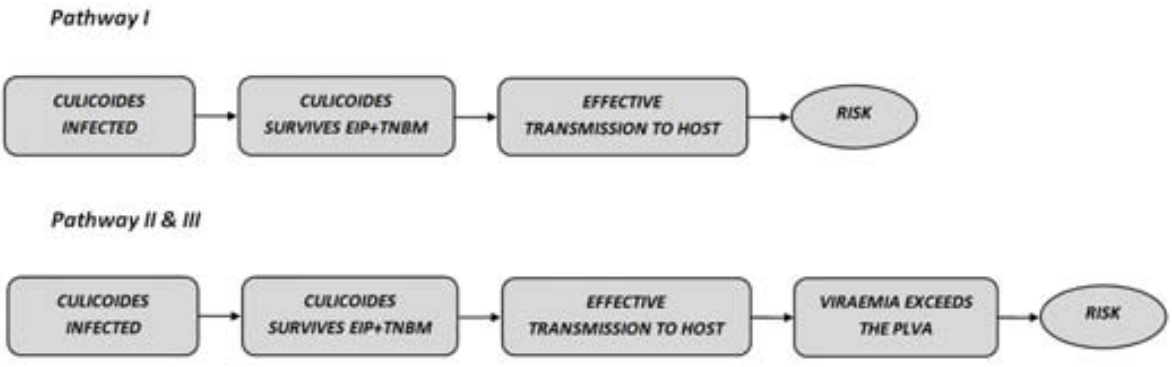

Figure 2: Steps for overwintering for pathway *I* and pathways *II* and *III*.

A detailed explanation of the model calculations for the different steps is available in the appendix.

3.1.2.3. Expert opinion workshop

Some parameters for which quantitative data were not available were estimated based on the opinion of experts. The method employed to elicit the opinion of experts was the Workshop Method, and was carried out during the First MedReoNet Annual meeting held in Palma of Majorca (Spain).

3.1.2.4. Modelling software

The spreadsheet model was constructed in Microsoft Excel (Microsoft® Office Professional Edition, 2003), and run for 50,000 iterations (Latin Hypercube sampling) in @Risk version 4.5.5 (© Palisade Corporation).

3.1.2.5. Sensitivity analysis

In order to identify those input parameters which were more influential in the model output(s), a sensitivity analysis was carried out. For each month, a regression analysis (either linear or logistic regression) was performed independently for the different steps in the transmission pathway: 1) Probability *Culicoides* getting infected, 2) Probability *Culicoides* survives *EIP & TNBM*, and 3) Probability of effective transmission. Furthermore, a second regression analysis to assess the influence of these steps in the final weighted probability was carried out. For these analyses, the results of each iteration of i) those input parameters which influenced these different steps (table I), ii) the probabilities associated to these steps, and also iii) the final weighted probability, were extracted from the model.

Outputs (Steps)	***Inputs***
Probability of *Culicoides* infection (per month)	Proportion of bites on cattle and on sheep Within farm prevalence in cattle Within farm prevalence in sheep Probability of viraemia month 0 to 3 in cattle and sheep Proportion of bites on infectious host that infect a midge Proportion of bites per infectious midge that infect a host Longevity of *Culicoides* (per month) Mean number of blood meals (per month)
Probability of surviving the *EIP* and the *TNBM* (per month)	Longevity of *Culicoides* (per month) Extrinsic Incubation Period (per month) Time to the Next Blood Meal (per month)
Probability of effective transmission	Proportion of bites on cattle and on sheep Proportion of bites per infectious midge that infect a host

Table I: Input parameters included in the sensitivity analysis of the different outputs

For quantitative outcomes, the relative strength of the input parameters was measured by the value of the standardized coefficient (beta). For categorical dichotomous outcomes, the

relative strength of the input parameters was measured by the values of the Wald estimate and the *exp(B)*.

The analyses were performed using SPSS 17.0.0 (Statistical Package for Social Sciences Inc., Chicago, IL, USA). A more detailed explanation of the sensitivity analysis is available in the appendix.

3.1.2.6. Scenario description

The model was applied to a real scenario: overwintering in Germany in 2006-2007. In 2006, BTV-8 was detected in Germany affecting 571 cattle farms and 309 sheep flocks. The region affected was mainly North Rhine-Westphalia, nearby the affected areas in Belgium, the Netherlands, and Luxembourg. Apparently, the infection overwintered in the region, and in 2007 spread over most of Germany (Conraths et al. 2009).

The specific inputs for the German scenario are shown in table II.

Description of model input parameter	**Value**		**Source**
Mean daily temperatures (ºC)	*Various (see figure 3)*		[1]
Monthly proportion of *Culicoides* captures during study period (November to April)	*Nov.: 0.977* *Dec.: 0.017* *Jan.: 0.002*	*Feb.: 0.001* *Mar.: 0.001* *Apr.: 0.001*	[5]
Monthly proportion of *Culicoides* captured outdoors (versus indoors)	*Nov.: 0.50* *Dec.: 0.40* *Jan.: 0.27*	*Feb.: 0.12* *Mar.: 0.32* *Apr.: 0.17*	[5]
Cattle population in North Rhine-Westphalia (H_c)	*1,346,488*		[2]
Sheep population in North Rhine-Westphalia (H_s)	*199,762*		[2]
Monthly cumulative incidence of cattle farms (CI_{ci})	*Aug. 2006: 1.8 x10^{-3}* *Sep. 2006: 3.0 x10^{-3}* *Oct. 2006: 1.4 x10^{-2}* *Nov. 2006: 8.5 x10^{-3}* *Dec. 2006: 2.0 x10^{-3}*	*Jan. 2007: 4.2 x10^{-3}* *Feb.2007: 2.7 x10^{-3}* *Mar. 2007: 1.0 x10^{-3}* *Apr. 2007: 1.6 x10^{-3}*	[2, 3]
Monthly cumulative incidence of sheep farms (CI_{si})	*Sep. 2006: 1.1 x10^{-2}* *Oct. 2006: 4.6 x10^{-2}* *Nov. 2006: 2.5 x10^{-2}* *Dec. 2006: 4.0 x10^{-3}*	*Jan. 2007: 0* *Feb. 2007: 0* *Mar. 2007: 0* *Apr. 2007: 0*	[2, 3]
Proportion of immune cattle	*0.01*		Model estimation[‡]
Proportion of immune sheep	*0.04*		Model estimation[‡]

Table II: Specific input parameters (Germany 2006-2007).

[1]Anonymous: Bundesministerium für Verkher, Bau und Stadtentwicklung. Klimadaten Deutschland. http://www.dwd.de/bvbw/appmanager/bvbw/dwdwwwDesktop? [consulted 6 August 2009]

[2]Anonymous: Statische Ämter des Bundes und der Länder. https://www.regionalstatistik.de/ [consulted 6 August 2009]

[3]Anonymous: EU. Food Safety Regulatory Committees: Standing Committee on the Food Chain and Animal Health (SCFCAH):http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations_en.htm#03042009 [consulted 8 August 2009]

[‡] The proportion of immune cattle and sheep were obtained based on the estimated number of cattle and sheep infected in 2006 (natural immunity) as vaccination did not start until 2008

Based on *Culicoides* catches in Germany a *PLVA* of 4 months (between January and April) was considered. The 2 months previous to the *PLVA* (November and December) were also considered for the analysis. The probabilities of overwintering by *Culicoides* emerged in each of these months were estimated. The mean daily temperatures in the area of study for the months considered (plus May) are represented in figure 3

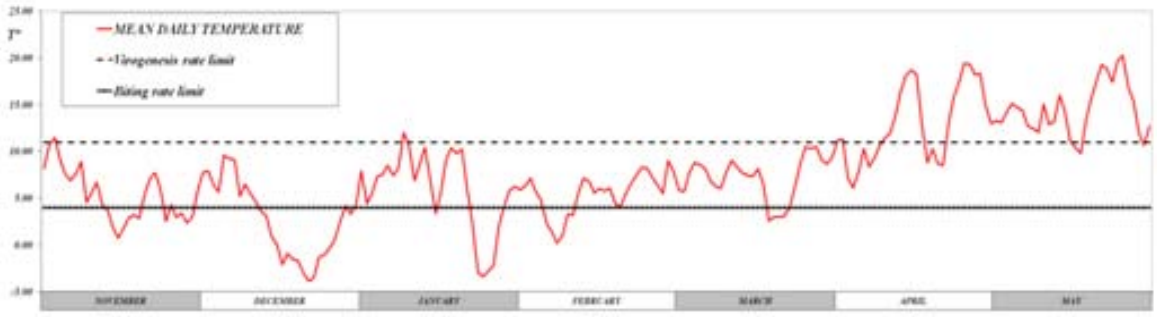

Figure 3: Mean daily temperatures (red line) for November to May in North Rhine-Westphalia. Virogenesis rate limit and biting rate limit are also shown. Source: Bundesministerium für Verkher, Bau und Stadtentwicklung. Klimadaten Deutschland. http://www.dwd.de/bvbw/appmanager/bvbw/dwdwwwDesktop?

The relative importance of the different pathways (*I, II & III*), and of overwintering by vectors infected before the start of the *PLVA* [a] or vectors infected during the *PLVA* [b], were assessed. Furthermore, the importance of the endophilic behaviour of *Culicoides* was also assessed by comparing the results i) assuming that all the vectors were subjected to the outside temperatures, and ii) assuming that the vectors had a certain probability of being inside, and therefore subjected to the inside temperatures. These probabilities were given by monthly proportion of *Culicoides* captured indoors versus outdoors (table II). The temperatures inside buildings were assumed not to vary widely because most of buildings in Northern Europe are likely to be closed, and the presence of animals contributes to the maintenance of the heat. Therefore, when outside temperatures were below 0ºC, inside temperatures were supposed to range between 10 and 15ºC, while when outside temperatures were above 0ºC, inside temperatures were supposed to range between 15 and 20ºC.

3.1.3. Results

The results are presented in 2 forms (table III):

- Per vector, i.e. given a vector which emerges in a given month, we estimated the probability it resulted in overwintering by each of the pathways considered. Results are presented both assuming exophilic behaviour exclusively and assuming that a proportion of vectors had endophilic behaviour.

- Weighted by the proportion of vectors which emerge in that month out of the total *Culicoides* emerged throughout the period of study. Differences were also made between exophilic behaviour exclusively and assuming that a proportion of vectors had endophilic behaviour.

PER VECTOR	*Probability Ia*		*Probability Ib*		*Probability IIIa*		*Probability IIIb*		*Total months*	
	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic
November	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
December	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
January	*NA*	*NA*	*0*	*0*	*NA*	*NA*	*0*	$1{,}2 \times 10^{-8}$	*0*	$1{,}2 \times 10^{-8}$
February	*NA*	*NA*	$5{,}9 \times 10^{-8}$	$5{,}5 \times 10^{-8}$	*NA*	*NA*	*0*	$6{,}7 \times 10^{-8}$	$5{,}9 \times 10^{-8}$	$1{,}2 \times 10^{-7}$
March	*NA*	*NA*	$9{,}2 \times 10^{-8}$	$8{,}7 \times 10^{-8}$	*NA*	*NA*	*0*	$2{,}1 \times 10^{-7}$	$9{,}2 \times 10^{-8}$	$3{,}0 \times 10^{-7}$
April	*NA*	*NA*	$1{,}1 \times 10^{-7}$	$1{,}6 \times 10^{-7}$	*NA*	*NA*	*0*	$5{,}1 \times 10^{-9}$	$1{,}1 \times 10^{-7}$	$1{,}6 \times 10^{-7}$
WEIGHTED RESULTS	*Probability Ia*		*Probability Ib*		*Probability IIIa*		*Probability IIIb*		*Total months*	
	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic	Exophilic	Endophilic
November	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
December	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
January	*NA*	*NA*	*0*	*0*	*NA*	*NA*	*0*	$1{,}4 \times 10^{-9}$	*0*	$1{,}4 \times 10^{-9}$
February	*NA*	*NA*	$1{,}6 \times 10^{-10}$	$6{,}2 \times 10^{-11}$	*NA*	*NA*	*0*	$1{,}3 \times 10^{-9}$	$1{,}6 \times 10^{-10}$	$1{,}3 \times 10^{-9}$
March	*NA*	*NA*	$1{,}2 \times 10^{-9}$	$1{,}6 \times 10^{-9}$	*NA*	*NA*	*0*	$3{,}6 \times 10^{-9}$	$1{,}2 \times 10^{-9}$	$5{,}1 \times 10^{-9}$
April	*NA*	*NA*	$9{,}4 \times 10^{-9}$	$2{,}3 \times 10^{-8}$	*NA*	*NA*	*0*	$1{,}8 \times 10^{-9}$	$9{,}4 \times 10^{-9}$	$2{,}5 \times 10^{-8}$
Mean	*0*	*0*	$1{,}1 \times 10^{-8}$	$2{,}4 \times 10^{-8}$	*0*	*0*	*0*	$8{,}0 \times 10^{-9}$	$1{,}1 \times 10^{-8}$	$3{,}2 \times 10^{-8}$

Table III: Results: Mean probabilities per vector for the different pathways and months of emergence of *Culicoides* given exophilic and endophilic behaviour. Weighted mean probabilities for the different pathways and months of emergence of *Culicoides* given exophilic and endophilic behaviour. Mean probabilities for the different months for pathway II were zero, and therefore are not shown in the table. NA: Not applicable.

The results per vector (table III) indicate that for exophilic *Culicoides* overwintering was only possible by vectors infected during the *PLVA* that infected the host after this period is finished (pathway *Ib*), and only by vectors that emerged after January, with the mean probabilities increasing between February (5.9×10^{-8}) and April (1.1×10^{-7}). Endophilic behaviour allowed transmission by both vectors infected during the *PLVA* that infect the host after this period is finished (pathway *Ib*) and by vectors infected during the *PLVA* that infect the host before this period is finished (pathway *IIIb*). This allowed advancing the period in which transmission was possible (to January). The mean probabilities of overwintering increased between January (1.2×10^{-8}) and April (1.6×10^{-7}). Overwintering by long term persistence in the ruminant host (pathway *II*) was not possible.

Of the steps considered in the pathways for overwintering (figure 2), the main determinants of the low probabilities obtained were the low likelihood of *Culicoides* infection and the low probability of *Culicoides* surviving the *EIP* and the *TNBM*. The probabilities of *Culicoides* infection for the different months were consistently higher for endophilic *Culicoides* as compared to exophilic (table IV), although the differences decreased gradually. Similarly,

endophilic behaviour increased the probabilities of surviving the *EIP* and the *TNBM* (table IV). The probabilities of effective transmission were always in the range of 0.9 and therefore did not have a great influence in the final result.

	Mean probability *Culicoides* infected		Mean probability *Culicoides* survives *EIP* + *TNBM*	
	Exophilic *Culicoides*	Endophilic *Culicoides*	Exophilic *Culicoides*	Endophilic *Culicoides*
November	4.1×10^{-5}	1.4×10^{-4}	0	1.4×10^{-3}
December	8.9×10^{-6}	4.0×10^{-5}	0	1.6×10^{-4}
January	1.4×10^{-5}	2.6×10^{-5}	0	2.4×10^{-4}
February	2.6×10^{-5}	4.1×10^{-5}	5.4×10^{-4}	1.8×10^{-3}
March	2.3×10^{-5}	2.8×10^{-5}	7.8×10^{-4}	2.3×10^{-3}
April	2.0×10^{-5}	2.0×10^{-5}	2.0×10^{-5}	2.0×10^{-5}

Table IV: Probabilities of *Culicoides* infection and probabilities of *Culicoides* surviving the *EIP* and *TNBM* for exophilic and endophilic *Culicoides* per month of emergence.

The sensitivity analysis showed that, for both the exophilic and endophilic scenarios, the most influential parameters in the probability of infection for the different months were the total number of blood meals, with mean values of the standardized coefficient (beta) of 0.57 and 0.68 for the exophilic and endophilic scenarios respectively; and the proportion of bites per infectious midge that infect a host, with mean values of beta of 0.37 and 0.31 for the exophilic and endophilic scenarios respectively. The longevity of *Culicoides* was eliminated from the regression model because of its statistically significant correlation to the number of blood meals, which was weaker in the case of endophilic *Culicoides*. For the probability of *Culicoides* surviving the *EIP* and the *TNBM*, the longevity of *Culicoides* was the most influential parameter (mean value of Wald statistic for both scenarios of 212). The values of *exp(B)*, that give the odds ratios, indicated that the longer a *Culicoides* live, the higher the probability it survives the *EIP* and the *TNBM*, although this increase was higher for exophilic *Culicoides* (mean *exp(B)* of 1.2 as compared to 1.1 for endophilic *Culicoides*). *TNBM* was also statistically significant, but the values of the Wald tests were much lower (mean value of 23 for both scenarios). The pattern of values of *exp(B)* is less clear, in general the shorter the *TNBM*, the higher the probability the *Culicoides* survives the *EIP* and the *TNBM*, but for some months in the exophilic scenario, the effect seemed to be the opposite. The *EIP* had to be eliminated from the regression model because of its statistically significant correlation with longevity. The only exception was for April in the endophilic scenario. The value of *exp(B)* indicated that the lower the *EIP*, the higher the probability the *Culicoides* survives the *EIP* and the *TNBM*. The most influential parameters in the probability of effective transmission was the proportion of bites per infectious midge that infect a host (beta=0.86), while the proportion of bites on cattle and on sheep (beta=0.51) seemed less important.

For exophilic *Culicoides* the mean weighted result (table III) was *1.1 x10^{-8}*, and almost 90% of the risk of overwintering was due to *Culicoides* emerged in April. For endophilic *Culicoides* the mean weighted results (table III), and a 78% of the risk was due to *Culicoides* emerged in April.

The assessment of the influence of the different steps in the final weighted probability indicated that by far the most influential step was the probability that *Culicoides* emerged in April survived the *EIP & TNBM* (beta = 0.34 and 0.40 for exophilic and endophilic *Culicoides* respectively). The second most influential step was that *Culicoides* emerged in March survived the *EIP & TNBM* (beta = 0.06 and 0.08 for exophilic and endophilic *Culicoides* respectively). The probability of infection of the *Culicoides* emerged in April was the third most determinant parameter (beta = 0.02 and 0.04 for exophilic and endophilic *Culicoides* respectively).

3.1.4. Discussion

In Germany, between 2006 and 2007, the length of the *PLVA* (4 months) did not allow overwintering by midges emerged before this period (pathways *Ia* and *IIIa*) neither with the exophilic nor with the endophilic behaviour. This long *PLVA* did not allow overwintering by hosts infected before the *PLVA* (pathway *II*) either.

For exophilic *Culicoides*, overwintering was only possible by pathway *Ib* as temperatures above the virogenesis rate limit were reached only a few days in April (figure 3), which did not allow the completion of the *EIP* and *TNBM*, and transmission to the host before the end of the *PLVA* (pathway *IIIb*). Endophilic behaviour appeared to favour overwintering mainly by increasing the probability by pathway *Ib*, and to a lesser extent by allowing the transmission of BTV to ruminants during the *PLVA* (pathway *IIIb*), which allowed advancing the period in which transmission was possible (to January). In fact, mild temperatures inside buildings did allow vectors emerged throughout the whole study period to survive the *EIP* and the *TNBM.* However, for vectors emerged in November and December, the duration of the *PLVA* (4 months) did not allow infected vectors (pathway *Ia*), or viraemic hosts (pathway *IIIa*) to reach May.

Overall, the sensitivity analysis highlighted the importance of the temperature-dependent parameters (longevity, *EIP* and *TNBM*) on the probability of BTV overwintering, although their relative importance was difficult to assess because of the correlation that exists among these parameters. The importance of longevity may be understood because of its influence in both the probability of infection and the probability of surviving the *EIP* and the *TNBM*. On the other hand, the duration of the *TNBM* seemed to have a less decisive role in the probability of overwintering, which might be explained by the fact that when temperatures were favourable for the completion of the *EIP*, they also allowed the rapid completion of the *TNBM*.

Of the non temperature-dependent parameters, the proportion of bites on an infectious host that infect a midge seemed to be the most influential. There is a great degree of uncertainty regarding this parameter as the distribution used was a combination of field estimates *C. sonorensis* and laboratory estimates for *C. obsoletus*, and variations in viral titres within the host and among different hosts, were not taken into account.

The results of the sensitivity analysis are in agreement with previous studies (Gubbins et al. 2008), and emphasize the need for further research in the estimation of these influential parameters.

Even though endophily seemed to favour overwintering, its effect was limited (the mean weighted probabilities were less than 3 times higher than for exophilic *Culicoides*). This is a consequence of the complex effect of temperature on BTV transmission: an increase of temperature reduces the duration of the *EIP* and the *TNBM*, but also the longevity of *Culicoides*; and a decrease of temperature increases the longevity of *Culicoides*, but also the duration of the *EIP* and the *TNBM*. Therefore, even though endophily (milder temperatures) increased the probability of vector infection (table IV), this probability is the result of the equilibrium between longevity and number of blood meals, and while endophily increased the number of blood meals in relation to exophily (lower temperatures), it also decreased longevity. Similarly, endophily increased the probability of surviving the *EIP* and the *TNBM* (table IV), but again, this probability is the result of the equilibrium between longevity and duration of the *EIP* and the *TNBM*, and while endophily decreased the duration of these 2 periods in relation to exophily, it also decreased longevity. This is somehow no unexpected because it is known that BTV transmission by *Culicoides* is inefficient, and that very few ever transmit the virus, so this has to be compensated by huge numbers of vectors (Wittmann & Baylis 2000). Given the low probabilities obtained for the pathways considered in the model, for these mechanisms to have played a major role in overwintering in Germany, the number of vectors present in winter would have had to be large. Even though *Culicoides* captured represent only a fraction of the *Culicoides* population, the number of *Culicoides* trapped during winter in Germany seems too small (captures during the *PLVA* represent only a 0.06% of the total of the year).

The low probabilities are consistent with what was observed in northern Europe, where the disease reappeared around areas of intense transmission rather than those where the transmission was most recent (EFSA 2008), and nearly all the northern European countries previously infected (Meiswinkel et al. 2008a). In fact, BTV isolation from overwintering populations of *Culicoides* has not been achieved yet (EFSA 2008). Therefore, other overwintering mechanisms not considered in the model seem to have played a decisive role in

overwintering in Germany. In 2008, transplacental transmission of field strains of BTV-8 was demonstrated in Northern Ireland (Menzies et al. 2008). Before this, it was thought only viruses passaged in tissue culture had the potential to cross the placenta, but since then, similar findings have been reported in several European countries (De Clercq et al. 2008; Darpel et al. 2009; Santman-Berends et al. 2010). However, whether PCR positive calves born to dams naturally infected during pregnancy are able to infect midges, and therefore play a role in overwintering is unknown (Darpel et al. 2009; Santman-Berends et al. 2010). Besides, mechanisms considered of minor significance during normal transmission, may become disproportionately important for the survival of the virus when normal transmission is interrupted by winter, and one or more of these mechanisms may be responsible for the cases of BTV transmission that have taken place during the winter in North-Western Europe (Wilson & Mellor 2009).

The model was applied to a given scenario, in this case Germany in 2006-2007 taking into account its specific conditions. Therefore, any conclusions drawn are specific of that scenario as different conditions (e.g. temperatures or duration of *PLVA*) may produce different results. In addition, different *Culicoides* species may differ in their ability to transmit BTV (Carpenter et al 2008; Gubbins et al. 2008). However, given the lack of species-specific data, all suspect and confirmed vector species were considered equally competent in transmitting all BTV serotypes, as recommended by EFSA (2008). In the proposed scenario (Germany), this is unlikely to have played a decisive role as *Culicoides obsoletus* was by far the most common species accounting for at least 70% of total captures, and more than 90% on some farms (Melhorn et al. 2009).

Only sheep and cattle were considered in the model. Even though goats are also susceptible to BTV, in the case of Germany, given the low number of goats, they are unlikely to have played an important role in BTV transmission. In fact, they constituted only a 0.35% of the infected domestic ruminants reported in Germany in 2007 (Conraths et al. 2009). In countries with larger goat populations (e.g. Southern European countries), they may need to be taken into account. Several species of wild ruminants are known to be susceptible to BTV infection, and in Germany BTV-8 has been detected in red deer, fallow deer, roe deer and mouflon (Conraths et al. 2009). However, the role played by these species on the epidemiology of BTV in Europe is difficult to predict. Other factors besides temperature, such as humidity may affect the transmission of BTV, as shown by Wittmann and collaborators (2002), but they were not taken into account because of the lack of data on the effect of humidity at different temperatures.

Besides, both variable and uncertain parameters were used, and that constrains the assessment of the relative contribution of variability and uncertainty on the results.

One advantage of the model is that it allows the estimation of the probabilities taking into account the specific conditions in a given country or area: i) pattern of *Culicoides* activity throughout the year, ii) bluetongue incidence in both bovine and ovine in the previous year, and iii) cattle and sheep populations. Furthermore, the model allows taking into account the effect of temperature in BTV transmission. Vectors are not maintained at constant temperatures and therefore the effect of daily variations in temperatures needs to be considered. In fact, it has been observed that in cool conditions orbiviruses may persist in vectors for long periods, and that subsequent exposure to warm temperatures resulted in replication of this latent virus allowing transmission (Wittmann et al. 2002).

The model provides a framework which may be useful for the assessment of the probability of overwintering of other vector-borne diseases, in particular other Orbiviruses such as Epizootic Hemorrhagic Disease (EHD) or African Horse Sickness (AHS).

3.2. STUDY II: Quantitative assessment of the risk of bluetongue by Culicoides introduced via transport and trade networks

3.2.1. Introduction

Bluetongue (BT) disease is caused by bluetongue virus (BTV), a member of the *Orbivirus* genus within the family Reoviridae, and affects both domestic and wild ruminants. It is included within the list of diseases notifiable to the OIE because of the impact on international trade of animals and animal products.

BT is transmitted almost exclusively via bites from adult females of certain species of *Culicoides* midges. Between 1998 and 2006, several BTV serotypes made incursions into Europe, but their distribution was limited to the Mediterranean Basin where the main Afro-Asiatic vector, *Culicoides imicola*, was present (Purse et al. 2005). However, in August 2006, a bluetongue outbreak, caused by bluetongue serotype 8 (BTV-8), was detected in the Netherlands from where it spread to Belgium, Germany, France and Luxemburg (Wilson & Mellor 2009), demonstrating the potential for Palearctic *Culicoides* species to sustain and propagate outbreaks (Carpenter et al. 2009a). The strain, which was of sub-Saharan origin (Maan et al. 2008) occurred 900 km further north than the northern latitudinal limit of previous European incursions and entirely bypassed southern Europe (Carpenter et al. 2009a). Investigations on the possible routes of introduction of BTV-8 in Northern Europe were carried out (Mintiens et al. 2008a). The most obvious mechanisms for BTV incursion into a free area, the importation of infected hosts or the transportation of infected *Culicoides* on airstreams seemed unlikely. No ruminants were imported from a third country with a known or suspected history of BTV incidence of any serotype or from a Member State with a known history of BTV-8, although introduction via illegal animal imports or by an intermediate stopover in another area could not be ruled out. On the other hand, the closest BTV affected area was far beyond the estimated distance travelled by *Culicoides* on wind, and in case of dispersion by wind one would have expected other locations in-between to be affected. Therefore, the introduction via other mechanisms, particularly, the potential for *Culicoides* to be imported along with or independently of the import of animals, plants or other 'materials', was also assessed (Mintiens et al. 2008a). In addition to ruminants, various non-susceptible mammal species may have acted as mechanical carriers of infected vectors. In fact, 31 horses originating from countries with a known or suspected history of BT had been transported into the area of first infection weeks before the start of the BTV-8 epidemic (Mintiens et al. 2008a). Besides, many insect species are associated with plants and consequently may be transported with them. This is not known to be the case for the haematophagous *Culicoides* vectors of BTV, which tend to associate much more closely with their mammalian hosts. However, as flowers exported from Africa into Europe are packed at night under bright artificial light, it was speculated that

infected *Culicoides* may have been included in the packing round the flowers. In addition, aircraft flights may theoretically transport infected *Culicoides*. Air, sea and land transport networks continue to expand and the pathogens and their vectors can now move further, faster and in greater numbers than ever before (Tatem et al. 2006). Though there are many records of insect species such as mosquitoes occurring in such situations, there appear to be almost no data recording the presence of *Culicoides* species on aircraft.

The introduction of BTV-8 into Western Europe was not a unique event, as in October 2008 BTV-6 was detected in the Netherlands and in January 2009 BTV-11 was detected in Belgium. Despite investigations, their route of introduction could not be identified either, and that impedes the implementation of measures to prevent the introduction of other serotypes (Wilson & Mellor 2009).

These events highlighted the fact that the potential for BTV introduction by means different to those usually involved. Further investigation of the risk for introduction of BTV-8 and other serotypes would be needed through an import risk assessment (Mintiens et al. 2008a).

The aim of this work was to assess, by means of a stochastic risk assessment model, the probability of development of a BTV outbreak as a consequence of the introduction of infected *Culicoides* via transport and trade networks. The model was applied to calculate the risk of development of a BTV-8 epidemic in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries (Germany, Belgium, the Netherlands, France, the Czech Republic, Denmark and the UK), regardless of the mechanism by which the midges were introduced. Luxemburg could not be assessed because of the lack of reliable data.

3.2.2. Materials and Methods

3.2.2.1. Model pathway

For a BTV outbreak in the country of destination to occur, a series of events (steps) have to take place (figure 1).

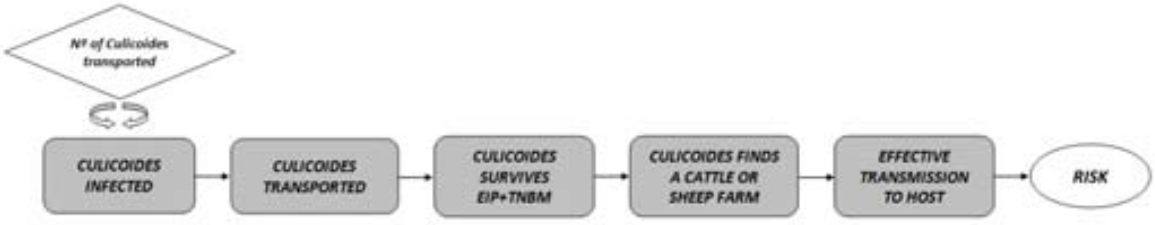

Figure 1: Pathway diagram with the steps considered for the estimation of the risk of BTV introduction by *Culicoides* introduced via transport and trade networks

Given a) the lack of data on the probability of *Culicoides* being carried in different means of transport or along with different traded materials, and b) the complexity of global transport and trade networks, it is impossible to estimate an overall risk.

However, the model allowed the estimation of the probability of a BTV outbreak in a particular country of destination by the introduction of a single *Culicoides* from a given country of origin. This probability was estimated taking into account a) the specific conditions in the country of origin: pattern of *Culicoides* activity and temperatures throughout the year, bluetongue incidence and the ruminant population, and b) the specific conditions in the country of destination: temperatures throughout the year and the ruminant population. Besides, the model allowed the estimation of the relative risk for the different months of the year.

3.2.2.2. Risk estimation

Some parameters for which quantitative data were not available were estimated based on the opinion of experts. The method employed to elicit the opinion of experts was the Workshop Method, and was carried out during the First MedReoNet Annual meeting held in Palma of Majorca (Spain) in December 2007.

The model pathway consisted of five steps (fig 1). Probabilities for steps 1 to 4 are not totally independent as vectors which live longer are more likely to get infected, to be transported, to survive the *EIP* and the *TNBM* and also to find a cattle or sheep farm in the country of destination. However, this fact was taken into account by using the value of longevity obtained in each iteration for the calculation of all these probabilities.

3.2.2.2.1. PROBABILITY OF A *CULICOIDES* GETTING INFECTED

Calculations as in study I (see appendix).

3.2.2.2.2. PROBABILITY *CULICOIDES* TRANSPORTED

Only those *Culicoides* which are transported after infection will pose a risk. Therefore, we firstly estimated the time left for *Culicoides* transportation (see figure 2) which was equivalent to the time from its infection to its death (*TITD*) as:

$$TITD = Longevity\text{-}TTCI$$

Where *TTCI* represented the time to *Culicoides* infection.

As *Culicoides* may be transported anytime between its emergence and its death, the probability the vectors are transported after they got infected (P_T) was estimated as:

$$P_T = TITD / Longevity$$

After infection, the day a *Culicoides* was transported was modelled by a *Uniform (0; TITD)* distribution, and the time left for BTV transmission (*y*) was estimated as *TITD – [Uniform (0; TITD)].*

Given their high variability in the duration of travels, the time of travel (in which transmission to a susceptible host would not occur), was not considered. Furthermore, conditions during travel (e.g. temperature) were assumed not to affect the viability of *Culicoides*.

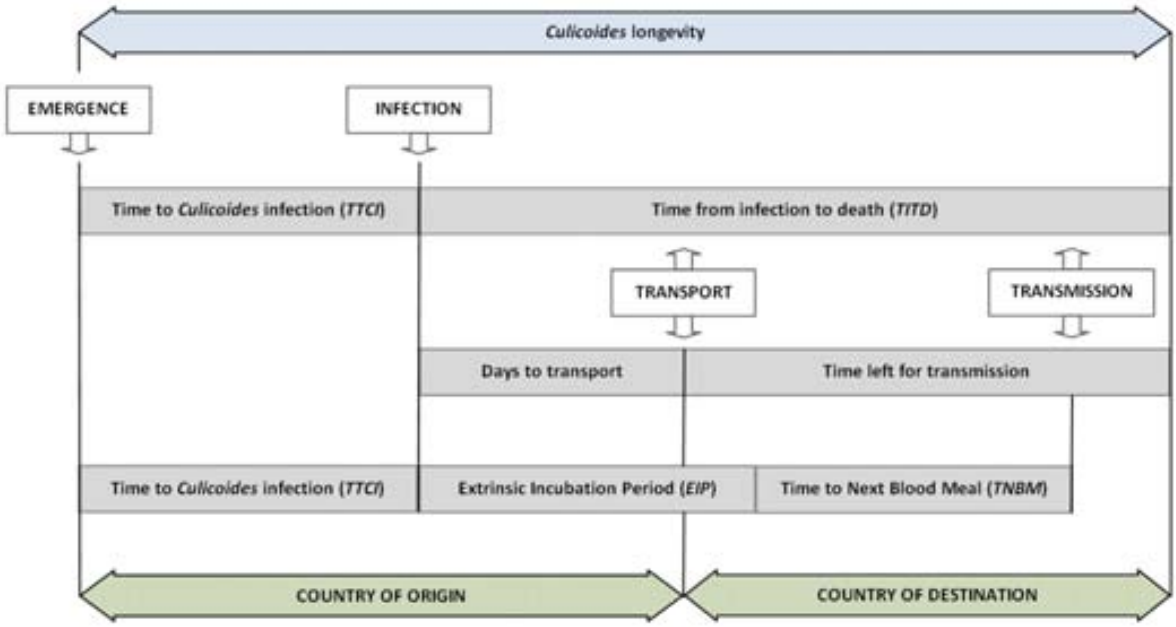

Figure 2: Diagram to estimate the probability of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries

After transportation, the daily temperatures that were used to estimate all the temperature-dependent parameters were those of the country of destination. Therefore, the adjusted longevity of *Culicoides* was estimated by using, after the day of transport of the vector, the daily probabilities of survival (P_S) estimated using the mean daily temperatures for the different days of the year in the country of destination.

3.2.2.2.3. PROBABILITY A *CULICOIDES* SURVIVES THE EXTRINSIC INCUBATION PERIOD (*EIP*) AND THE TIME TO THE NEXT BLOOD MEAL (*TNBM*)

Calculations as in study I (see appendix).

3.2.2.2.4. PROBABILITY OF A *CULICOIDES* FINDING A CATTLE OR SHEEP FARM IN THE COUNTRY OF DESTINATION

For BT introduction into a free area or country, the infected vector needs to be able to find a cattle or sheep farm. The probability of finding a farm is dependent on:

a) The distance travelled by a *Culicoides* in a single day: a value of 2 Km/day, as reported by Mellor (Mellor et al. 2000) was assumed, which is consistent with the rate of spread previously reported in Sardinia (Gerbier et al. 2008).

b) The range of detection: Defined as the (lateral) distance at which the *Culicoides* will be able to detect a new host. Biting insects have evolved a complex sensory system designed to detect and locate vertebrate hosts for blood feeding (Grant & Kline 2003). *Culicoides* respond to a wide range of host-derived kairomones (Bhasin et al. 2001). Also carbon dioxide is involved in orientation of *Culicoides* to host animals (Bhasin et al. 2000). Besides, female *C. impunctatus* produce a volatile "invitation pheromone", which recruits females to a host (Bhasin et al. 2001). These olfactory stimuli complement visual cues for midge attraction to a host (Bhasin et al. 2001). Heat may also play a role, as collection of *Culicoides* in CDC-type traps were significantly increased with the addition of heat (Kline & Lemire 1995).

The range of detection assumed is derived from the work by de Koeijer and collaborators in the Netherlands (de Koeijer et al. 2007):

1- They estimated that the traps used attracted midges from a range of about 25 meters around the trap.

2- They used a black light trap, which seems to be much more attractive to midges than CO_2 traps. As CO_2 traps are supposed to simulate one host, the value for the model is obtained from a uniform distribution between 0 and 25.

The distance travelled by a *Culicoides* in a single day and the range of detection define the area covered by one *Culicoides* in a single day. If a (sheep or cattle) farm was present in this area it was assumed to be found by the vector.

The daily probability of finding a farm (P_{f1}) was therefore estimated taking into account:

a- The area covered by one *Culicoides* in a single day (Km^2/day).

b- The farm (cattle + sheep) density (per square Km) in the country of destination. For simplification purposes, farms were assumed to be homogenously distributed.

Then, taking into account the *y* days left for BTV transmission (see 3.2.2.2.2.), the final probability of finding a farm (P_F) was calculated as:

$$P_F = [1 - (1 - P_{f1})^{y}]$$

This probability (P_F) was estimated independently for cattle and for sheep because the probabilities of BTV transmission to cattle and to sheep are different.

3.2.2.2.5. PROBABILITY OF EFFECTIVE TRANSMISSION

Estimated as in study I (see appendix).

3.2.2.3. Modelling software

The spreadsheet model was constructed in Microsoft Excel (Microsoft® Office 2007, Redmond, WA), and run for 20,000 iterations (Latin Hypercube sampling) in @Risk version 5.5.0. (© Palisade Corporation, Ithaca, NY). This allowed the convergence of all the output probability distributions. The sensitivity analysis was performed using @Risk version 5.5.0.

3.2.2.4. Scenario description

The model was used to estimate the probability of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries (Germany, Belgium, the Netherlands, France, the Czech Republic, Denmark and the UK), regardless of the mechanism by which the midge was introduced.

Probabilities were estimated for each month in 2007 taking into account the conditions in the country of origin (mean daily temperatures, cattle and sheep populations and number of outbreaks in cattle and sheep), as well as in the country of destination, i.e. Spain (mean daily temperatures and cattle and sheep populations). For each country, mean daily temperatures for 2007 were obtained. In order to do that, mean daily temperatures for all the weather stations available in the country were extracted from the European Climate Assessment & Dataset (ECA&D) project website (http://eca.knmi.nl/index.php), and the mean value for the different locations was used for model calculations. The mean daily temperatures in different countries of origin (Belgium, the Netherlands, Germany and France), plus the country of destination (Spain) for 2007 are presented in figure 3.

Figure 3: Mean daily temperatures in different countries of origin (Belgium, the Netherlands, Germany and France), plus the country of destination (Spain) for 2007.

The monthly probabilities were later combined taking into account the proportion of *Culicoides* hatched each month to produce the weighted annual risk.

3.2.3. Results

The weighted annual risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of a single infected *Culicoides* from the affected Northern European countries varied significantly depending on the country from which the vector originated (figure 4). The highest risk was derived from *Culicoides* imported from Belgium ($3.2x10^{-7}$), the Netherlands ($2.6x10^{-7}$), Germany ($1.9x10^{-7}$) and France ($6.1x10^{-8}$), while the risk by *Culicoides* imported from the remaining countries was much lower Denmark ($4.3x10^{-11}$), the UK ($3.8x10^{-10}$), Switzerland ($2.2x10^{-11}$) and the Czech Republic ($6.4x10^{-12}$).

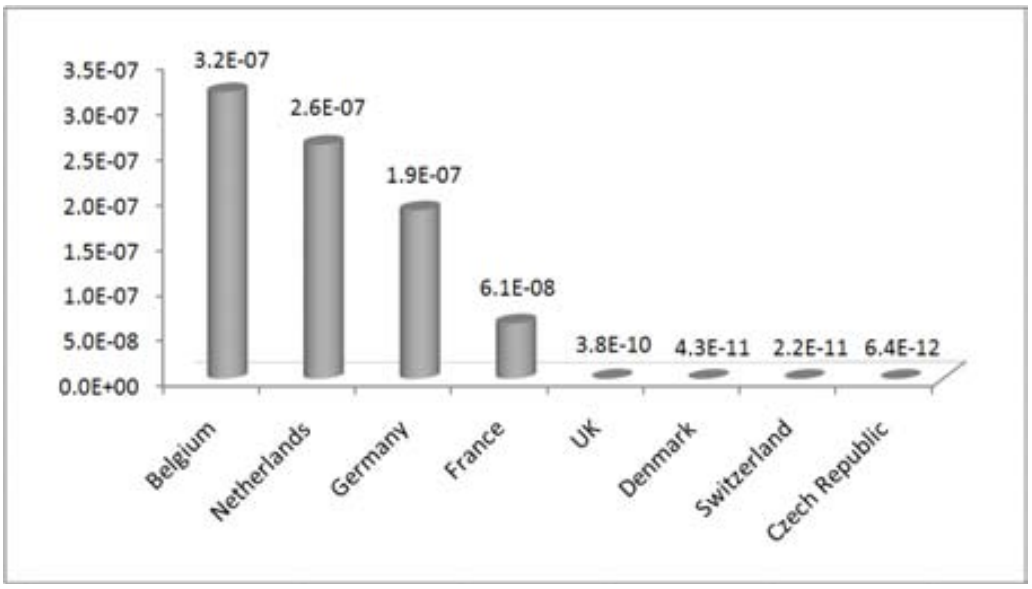

Figure 4: Weighted annual risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries.

The probability of a BTV-8 outbreak in Spain had an important seasonal component (table I). In general, the risk of a BTV-8 epidemic in Spain before April/May seemed almost negligible, then started to rise to reach a peak by September/October, then decreased to disappear after December 2007/January 2008. Only *Culicoides* hatched between January and December 2007 were considered for model calculations. However, for some of the countries of origin, *Culicoides* hatched at the end of 2007 were able to get infected and be transported, but the transmission to a susceptible host occurred actually in 2008 (January).

	January 2007 to April 2007	May 2007	June 2007	July 2007	August 2007	September 2007	October 2007	November 2007	December 2007	January 2008	February 2008
Germany	0.0E+00	1.7E-09	9.6E-10	1.8E-08	1.6E-07	3.4E-07	5.2E-07	1.1E-07	5.7E-09	2.3E-09	0.0E+00
Belgium	0.0E+00	0.0E+00	1.3E-08	1.5E-07	4.1E-07	6.5E-07	5.9E-07	1.2E-07	1.2E-08	0.0E+00	0.0E+00
France	0.0E+00	0.0E+00	1.4E-11	1.8E-09	3.4E-08	1.1E-07	2.2E-07	7.0E-08	0.0E+00	1.3E-09	0.0E+00
Netherlands	0.0E+00	1.1E-09	2.0E-09	2.4E-08	2.5E-07	4.1E-07	9.1E-07	1.1E-07	2.4E-08	0.0E+00	0.0E+00
Denmark	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	8.9E-11	2.1E-10	4.0E-11	0.0E+00	0.0E+00	0.0E+00
UK	0.0E+00	0.0E+00	0.0E+00	0.0E+00	5.7E-11	7.2E-10	2.0E-09	1.9E-10	8.0E-12	2.1E-11	0.0E+00
Switzerland	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.3E-11	1.1E-10	3.8E-11	1.2E-11	0.0E+00	0.0E+00
Czech Republic	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.3E-11	9.2E-12	0.0E+00	0.0E+00

Table I: Monthly risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries

The probabilities for the different steps in the transmission pathway for the different months of emergence of *Culicoides* in Belgium are presented in table II. Of these steps, the probability of *Culicoides* infection and the probability it survived the *EIP* and the *TNBM* were the most influential on the final probability (BTV-8 outbreak in Spain). In Belgium, the probability of *Culicoides* infection increased gradually from January, it reached a peak in August, and then progressively decreased. On the other hand, the survival of the *EIP* and the *TNBM* was only possible between May and October.

BELGIUM	January	February	March	April	May	June	July	August	September	October	November	December
Probability of infection	4.0E-09	6.7E-09	6.5E-08	3.2E-07	5.5E-06	9.3E-05	3.8E-04	8.0E-04	6.8E-04	2.3E-04	4.4E-05	7.4E-06
Probability of surviving the EIP+TNBM	0.0E+00	0.0E+00	0.0E+00	0.0E+00	8.8E-03	1.1E-02	7.5E-03	9.7E-03	9.8E-03	5.3E-05	0.0E+00	0.0E+00
Probability Culicoides transported	3.5E-02	2.9E-02	3.9E-02	7.4E-02	2.8E-01	5.6E-01	5.9E-01	6.1E-01	6.1E-01	6.5E-01	6.3E-01	7.5E-01
Probability of finding a host	0.0E+00	0.0E+00	0.0E+00	6.6E-04	2.4E-02	3.5E-02	2.6E-02	2.7E-02	4.8E-02	1.1E-01	1.6E-01	1.8E-01
Probability of effective transmission	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01	8.7E-01
Final probability	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.2E-09	2.1E-08	2.2E-07	7.6E-07	9.4E-07	4.4E-09	0.0E+00	0.0E+00

Table II: Probabilities for the different steps in the transmission pathway for the different months of emergence of *Culicoides* in Belgium

The sensitivity analysis showed that the most determinant parameters in the risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of an infected *Culicoides* from the affected Northern European countries were the longevities of *Culicoides* in the months in which BTV transmission was most intense (results not shown).

3.2.4. Discussion

Air, sea and land transport networks continue to expand in reach speed and volume. Aviation, in particular, has expanded rapidly and passenger numbers have grown at nearly 9% per year since 1960 and are expected to continue to increase. Similarly, globalization of the world economy has resulted in an important increase of shipping traffic (Tatem et al. 2006). One of the important consequences of global transport network expansion is vector-borne pathogen importation. Even though the introduction of insects and their pathogens via ships and aircraft is well documented (Gratz et al. 2000; Lounibos 2002), *Culicoides* have been mainly overlooked because of its small size, fragile nature and specialist taxonomy, and therefore the data available is quite limited (Carpenter et al. 2009a). Reye (Reye 1964) reported a probable spread of *Culicoides* by aircraft from Fiji to the Society Islands. Nie and collaborators (2005) found *Culicoides* in 9 out of the 70 ships inspected at Qinhuangdao port, China. In fact, some countries, concerned with this issue have implemented surveillance for *Culicoides* at border controls, e.g. the Australian Quarantine and Inspection Service (Carpenter et al. 2009a). In July 2003, one single individual of *Culicoides imicola* was caught in a trap at latitude 46°N, in the Ticino region (Cagienard et al. 2006b), which was very surprising given that the previous northernmost record of *C. imicola* presence in Europe was at 42°18'N in Spain (Sarto i Monteys et al. 2005). Because of the proximity to the Lugano airport, one of the considered scenarios to account for the finding was the introduction via airplanes, even though *C. imicola* does not seem to enter confined buildings and feeds exclusively on animals (Nevill 1978; Mellor et al. 2000).

The weighted annual risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of a single *Culicoides* from the affected Northern European countries seemed to be low (between 3.2×10^{-7} and 6.4×10^{-12}), although there were significant differences among countries. The low risk is consistent with the known fact that the probability of an individual *Culicoides* being able to transmit BTV is low as a consequence of the low probabilities that a *Culicoides* will: a) feed on a viraemic host, b) be competent to transmit the virus, c) survive the *EIP* and d) subsequently feed on a susceptible host (Wittmann & Baylis 2000). The highest weighted annual risks were by *Culicoides* imported from Belgium, the Netherlands, Germany and France, which, as there were no significant differences in temperature among countries, was mainly a consequence of the fact that these countries had the highest proportion of affected farms, while the risk by *Culicoides* imported from Denmark, the UK, Switzerland and the Czech Republic was much lower because the proportion of farms affected in those countries was low.

The risk of a BTV-8 outbreak in Spain because of the transportation of *Culicoides* from the majority of countries peaked in October, while for *Culicoides* from Belgium peaked in September, and from the Czech Republic in November. These peaks were mainly a consequence of the moment in which the incidence of BTV infection of farms in the country of origin reached the maximum value, but were also dependent on the temperatures in the country of origin and destination, which determined the time needed for the infection of the vector and the transmission to a susceptible host.

The results for the different steps in the transmission pathway (depending on the month of emergence of *Culicoides* in Belgium) indicate that the months in which BTV transmission was not possible this was due to the fact that vectors were not able to survive the *EIP* and the *TNBM*. This is the consequence of the restrictive conditions in relation to temperature required for virogenesis, as for the completion of the *EIP*, mean daily temperatures need to consistently reach values above 11^{o}C. For the months in which BTV transmission was possible, the probability of a *Culicoides* getting infected was the most determinant factor for the low overall probability obtained. This is the consequence of on the one hand the low monthly probabilities of the hosts being viraemic, and on the other the low proportion of bites on an infectious host that infect a midge.

The sensitivity analysis identified the longevities of *Culicoides* in the months in which BTV transmission was more intense as the most influential parameters in the risk of a BTV-8 outbreak in Spain in 2007. This is a reflection of the fact that the longevity of *Culicoides* influences the probabilities for the first 4 steps in the transmission pathway (probabilities of infection, survival of the *EIP*+*TNBM*, transport and finding of a susceptible host).

For many parameters (e.g. monthly incidence) data was only available at country level, and therefore probabilities were estimated for each (importing) country. However, within a country parameters may vary widely, and that may affect the results of the model. In order to calculate the weighted annual risk, data on the proportion of *Culicoides* caught each month is needed. However, this type of information was only available for different areas of Germany (Hörbrand & Geier 2009; Clausen et al. 2009), and therefore a common pattern of *Culicoides* activity (for Northern European countries) had to be used.

The model calculations are based on the assumption that the mortality of *Culicoides* does not increase as a consequence of transport. The OIE (OIE 2007a) includes vector control, especially in aircraft, as sanitary prophylaxis to prevent incursion of BTV in disease-free areas. Systematically spraying of aircrafts with insecticides might significantly reduce the risk of vectors surviving the transport, although whether or not this practice is common is not known.

An initial difficulty in defining the risk of a BT incursion lies in assessing the frequency and mechanism of introduction of pathogens or pathogen-infected hosts into an area, together with their associated probability of onwards transmission. To date, the best-characterized mechanisms for BTV incursion are via the movement of viraemic hosts or animal products from affected areas or via dispersal of infected *Culicoides* on airstreams (Carpenter et al. 2009a). One largely unaddressed aspect of BT epidemiology has been the potential for movement of infected adults *Culicoides* via local and global transportation networks (Carpenter et al. 2009a), and therefore the risk had to be estimated per vector. That is, given a *Culicoides* hatched in month *i* in country *c*, the probability of emergence of a BTV-8 epidemic in Spain assuming that a vector was transported (by any of the possible means), was calculated. For the calculation of the actual probability of a BTV outbreak in a given country, further research on the probability of *Culicoides* transportation via different transport and trade networks, or the effect of transport on vector survival would be needed. However, it is clear that given that the probabilities per vector are low, for this mechanism to pose a significant risk to BTV-free countries, the number of vectors transported would have to be very large.

The model may be also applied to assess the risk derived from the introduction of other Orbiviruses (e.g. African horse sickness virus or Epizootic hemorrhagic disease virus), or could be even be used as a framework for other vector-borne diseases. It may also be combined with wind-borne spread models to estimate the risk of BTV outbreaks by *Culicoides* transported on the wind.

3.3. STUDY III: Quantitative assessment of the probability of bluetongue virus transmission by bovine semen and effectiveness of preventive measures

3.3.1. Introduction

BT is an infectious viral disease of ruminants transmitted by the bites of *Culicoides* midges, and is caused by BTV, which belongs to the family *Reoviridae*, genus *Orbivirus*. BT is a World Organization for Animal Health (OIE) reportable disease of considerable socioeconomic impact and of major importance in the international trade of animals and animal products (Saegerman et al. 2008).

The emergence of BT in a new area may occur as a consequence of the introduction of infected *Culicoides* carried by various living organisms (plants, animals) or inanimate means (airplanes, ships); through active flight of infected vectors; or through passive transport of infected *Culicoides* on the wind. The introduction of BT may also occur through the movement of susceptible animals or animal products, including semen (Saegerman et al. 2008). In fact, semen transmission was one of the hypotheses for the introduction of BTV-8 into north-western Europe in 2006 (Mintiens et al. 2008a).

Artificial insemination in cattle is vital to the genetic improvement of the cattle population, and as a consequence millions of semen doses are produced annually worldwide (Thibier & Wagner 2002). Besides, international movements of semen have increased dramatically, so worldwide semen exchange requires particular attention to animal health aspects (Thibier & Wagner 2002). The hypothesis that infected bulls could excrete BTV in their semen led to restrictions on the international trade of ruminant semen and the establishment of measures to prevent BTV transmission by semen. However, neither the risk of BTV transmission by semen nor the efficacy of these measures had ever been estimated quantitatively. The Sanitary and Phytosanitary Agreement of the World Trade Organization requires the member states to base the regulation of the international movement and trade of animals and animal products on objective risk assessment (WTO 1995).

Given on the one hand the considerable expansion of BT in recent years (Wilson & Mellor 2009), and on the other the huge number of doses of bovine semen produced worldwide and the increase in the international movement of semen (Thibier & Wagner 2002), the transmission of BTV by semen could have devastating consequences in the cattle industry worldwide. In the European Union (EU), Council Directive 2000/75/EC (Anonymous 2000) lays down control measures to combat BT including a ban on the movement of semen from a semen collection centre (SCC) located in a restricted zone. However, Commission Regulation 1266/2007/EC (Anonymous 2007) includes the measures that may be applied for exemption of semen from the exit ban. These measures, which are aimed at preventing transmission by semen of animals from a SCC located in an endemic area, are based on those contained in article 2.2.13 of the OIE Terrestrial Animal Health Code (OIE 2007b).

Annex III of Commission Regulation 1266/2007/EC (Anonymous 2007) includes the conditions for exemption from movement restrictions, if any of the measures available to prevent BTV transmission by semen are applied. Semen may have been obtained from donor animals which for at least 60 days before commencement of and during collection of the semen have been: a) kept outside a restricted zone, b) protected against vectors or c) kept during the seasonally vector-free period in a BT seasonally-free area. Donor animals may also be subjected to diagnostic tests according to the OIE Terrestrial Manual, with negative results: either serological tests every 60 days or PCR tests at least every 28 days.

The risk reduction achieved by some of these measures was assessed. Scenarios (a) and (c) could not be assessed because they are highly unpredictable. If animals were kept outside a restricted zone, the risk of BTV infection would be theoretically zero. However, as demonstrated in Belgium, new cases do appear outside restricted areas, and the probability of detection depends on factors such as awareness of farmers or effectiveness of surveillance and control in that area (Mintiens et al. 2008b). Similarly, if animals are kept during the seasonally vector-free period in a BT seasonally-free zone, the risk of BTV infection would also be theoretically zero. However, in contrast to southern Europe, in the rest of Europe, a period of total cessation of activity of the adult vectors seems not to occur (Saegerman et al. 2008). With regard to scenario (b), the risk reduction achieved by the use of insecticides (regardless of whether they increase the vector mortality rate, decrease the biting rate or both) would be proportional to the reduction of the probability of the hosts (donor bulls) getting infected. However, actual estimation of this risk reduction is not possible with the available data (EFSA 2008).

The aims of this study were to assess, in case of introduction of BTV into a bovine SCC, both the risk of BTV transmission by semen and the risk reduction achieved by some of the preventive measures available. In order to achieve this, a stochastic risk assessment model was constructed. The model was applied to different scenarios, constructed according to: a) the type of diagnostic test and interval between the controls of donor bulls (either ELISA every 60 days or PCR every 28 days), b) the rate of BTV spread within the SCC (either low or high), and c) the timing of tests (either simultaneous or non-simultaneous). Besides testing of donor bulls, another general approach to prevent disease transmission by semen would be testing the end product (Wentink et al. 2000). Therefore, the effectiveness of testing the semen samples was also assessed. The results were compared with the probability of transmission if no control measures were applied.

3.3.2. Materials and Methods

3.3.2.1. Risk assessment model

The model calculates the probability of BTV transmission by semen by simulating individual bulls.

3.3.2.1.1. Estimation of monthly probabilities of BTV transmission by semen (P_{Tm})

The longer the time passing since infection of the SCC, the higher the probability of a donor bull being infected and viraemic, but also the higher the probability of detection of an infected animal. In order to better characterize the temporal pattern in the probability of BTV transmission by semen, probabilities were estimated independently considering whether the semen sample was collected within the first month after infection of the SCC (*month 1*), between the first and the second month after infection of the SCC (*month 2*), and so on.

The model estimates the probability of BTV transmission per semen dose, given infection of the SCC. A diagram of model calculations and steps in the transmission pathway is shown in figure 1. Values, abbreviations and sources of data of model input parameters are presented in table 1.

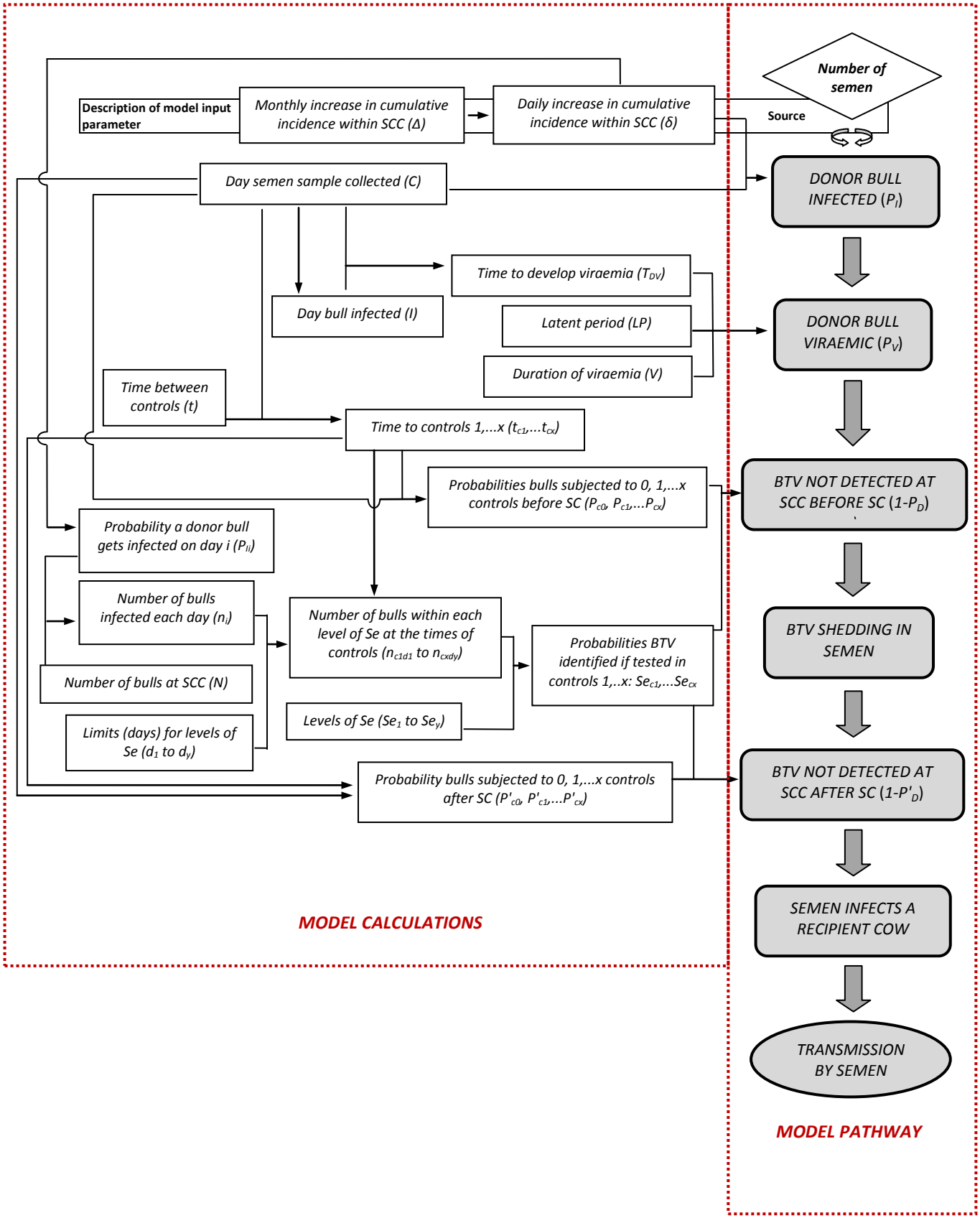

Figure 1: Diagram of model calculations and steps in transmission pathway for the estimation of the probability of BTV transmission (per semen dose for a given month). SC: Semen collected

Latent period (days)	*LP*	*Uniform (7,14)*	(Boneau et al. 2002)
Duration of viraemia (days)	*V*	*Gamma (5,4.12)*	(Santman-Berends et al. 2010)
Sensitivity of ELISA	Se_{ELISA}	*0.00 (from 0-7 days p.i.)* *0.25 (from 7-9 days p.i.)* *0.50 (from 9-11 days p.i.)* *0.70 (from 11-15 days p.i.)* *0.75 (from 15-20 days p.i.)* *0.90 (from 20-60 days p.i.)* *0.99 (from 60-79 days p.i.)* *1.00 (from 80 days p.i.)*	(EFSA 2008), based on: (Reddington et al. 1991; Afshar et al. 1992; Afshar et al. 1993; Zhou et al. 2000)
Sensitivity of PCR	Se_{PCR}	*0.00 (from 0-3 days p.i.)* *0.995 (from 3 days p.i.)*	(Vandenbussche et al. 2008)
Probability of BTV shedding in semen of viraemic bulls (Low-risk serotypes)		*Beta (1,232)*	Based on: (Gard et al. 1989; Kirkland et al. 2004)
Probability of BTV shedding in semen of viraemic bulls (BTV-8)		*Beta ([Uniform (48,78)+1], [89-[Uniform (48,78)+1]+1]) x Beta (5, 6)*	(Vanbinst et al. 2010)
Probability semen infects recipient cow		*Beta (4,7)*	(Bowen & Howard 1984)

Table 1: Model input parameters: Values, abbreviations and sources of data (p.i: post-infection)

3.3.2.1.1.1. <u>Probability of a donor bull being infected at the time of semen collection</u>

The probability of a donor bull being infected at any time point after the introduction of BTV in a SCC is dependent on the rate at which BTV spreads within that SCC. Because no specific measures to prevent BTV transmission are applied in a SCC, the spread was assumed to occur as within a cattle farm. The rate of BTV spread within a farm varies depending on factors such as the season or the area in which the farm is located (Santman-Berends et al. 2010). In order to assess the effect of the level of BTV spread on the risk of transmission by semen, two different levels were assumed: a) low: a monthly increase in cumulative incidence of 5% (percentage points), and b) high: a monthly increase in cumulative incidence of 25% (percentage points). BTV transmission relies mainly on the presence of favourable conditions for the activity of vectors, and therefore occurs only during certain periods during the year. A period of transmission within the SCC of 2 months was assumed (Santman-Berends et al. 2010), after which no animal would become infected. There is a great variability in the daily rate of BTV spread depending on several factors. The temperature is the most determinant one, and that is a reflection of all the temperature-dependent parameters in BTV spread (i.e., biting rate, extrinsic incubation period or mortality rate) (Gubbins et al. 2008), but other factors such as humidity (Wittmann et al. 2002) or even wind velocity (De Deken et al. 2008) may affect the daily transmission rate. However, for simplification purposes, within the 2-months transmission period, a linear increase in the daily cumulative incidence within the SCC was assumed.

Probability calculations

Given a monthly increase in cumulative incidence within the SCC (*Δ*), the daily increase in cumulative incidence within the SCC (*δ*) was calculated as:

$$\delta = \frac{\Delta}{30}$$

The day on which the semen sample was collected (*C*) for a given month was drawn from a *Uniform* distribution between the start and the end of that month (i.e., *Uniform (0,30)* for month 1, *Uniform (30,60)* for month 2, and so on).

Therefore, the probability of a donor bull being infected at the time of semen collection (P_I) was calculated as:

$$P_I = \delta \times C$$

3.3.2.1.1.2. Probability of a donor bull being viraemic at the time of semen collection

Probability calculations

The day on which a donor bull (from which the semen sample was obtained) became infected (*I*) was drawn from a *Uniform (0,C)* distribution.

After the latent period (*LP*) (i.e., the period from the infection of the bull until the start of viraemia), which was drawn from a *Uniform* distribution between 7 and 14 days (Bonneau et al. 2002), the animal developed a viraemia (*V*) with a duration in days given by *Gamma (5, 4.12)* (Gubbins et al. 2008).

If we calculate the time the animal has to develop the viraemia (T_{DV}) as *C* minus *I*, the probability of a donor bull being viraemic when the semen sample was collected would be given by the probability that two conditions were fulfilled:

a) Viraemia had started ($T_{DV} > LP$)

b) Viraemia had not ended ($T_{DV} < [LP + V]$)

3.3.2.1.1.3. Probability of BTV not being detected at the SCC (before semen was collected)

For the estimation of this probability, all bulls at the SCC have to be considered (i.e., if any donor bull at that SCC was found to be infected by BTV, it was assumed that no movement of semen from that SCC would be allowed). Donor animals may be subjected to either ELISA tests every 60 days or PCR tests every 28 days.

Probability calculations

For each month after infection of the SCC (*month 1, month 2,..*), the probability of detection at this centre is dependent on a series of factors:

A- The time available for BTV detection before the semen sample was collected: Given by the time between the introduction of BTV in the SCC (time 0) and the day in which the semen sample was collected, and therefore is equal to *C*.

B- The interval between controls: Given an interval between controls of *t* days (60 days in the case of serological tests and 28 days in the case of PCR), the time to the first control (t_{c1}) was drawn from a *Uniform (0, t)* distribution, the time to the second control (t_{c2}) was calculated as [t_{c1}+ *t*], and so on. If $C < t_{c1}$, the bulls at the SCC were not subjected to any control before the collection of semen. If $C > t_{c1}$, the bulls at the SCC were subjected to a control before the collection of semen (at time t_{c1}). If $C > t_{c2}$, the bulls at the SCC were also subjected to a second control before the collection of semen (at time t_{c2}), and so on.

Therefore, the probabilities that the bulls were controlled at times t_{c1},.., t_{cx} (P_{c1},.., P_{cx}) would be given by the probabilities that $C > t_{c1}$,.., t_{cx}.

C- The rate of BTV spread within the SCC, which determines the number of bulls infected each day during the transmission period.

D- The sensitivity of the diagnostic tests: The sensitivity (*Se*) of the diagnostic tests for BT (either ELISA or PCR) is not a fixed value, but depends on the time since the infection of the animal (EFSA 2008). Therefore, for each of these tests, different levels of sensitivity were considered depending on the time since infection, e.g., for an animal infected for less than d_1 days, the corresponding sensitivity would be given by Se_{d1}, for an animal infected between d_1 and d_2 days by Se_{d2}, and for an animal infected between d_{y-1} and d_y by Se_{dy}.

The values of sensitivity of the ELISA as a function of time (table 1) were obtained from the EFSA report (2008), based on several studies (Reddington et al. 1991; Afshar et al. 1992; Afshar et al. 1993; Zhou et al. 2000). For the PCR, it was assumed that the sensitivity was zero for the first 3 days and 99.5% thereafter (Vandenbussche et al. 2008). Even though the level of RNA in the blood wanes with time, RNA may persist at low levels for up to 200 days post-infection (Batten et al. 2009).

E- The number of animals controlled: The number of animals in the SCC influences the number of semen doses produced, but also the probability of detection, as this is estimated considering all the animals in the centre. For the model calculations, 20 donor bulls were assumed to be present in the SCC, although the effect of the number of animals was also evaluated.

F- The timing of controls: The time required between controls is defined in the legislation. However, differences in the probability of BTV detection may arise depending on whether the animals in the SCC are tested all at the same time (simultaneous testing) or randomly (non-simultaneous testing).

Probability calculations

I- Simultaneous testing

Given the daily increase in cumulative incidence within the SCC (δ), the probability of a donor bull being infected on day i (P_{Ii}) was calculated as:

$$P_{Ii} = \delta \times i$$

For i =1 to 60 days (the assumed duration of the transmission period).

Given the number of bulls in the SCC (N), the number of bulls becoming infected each of the days during the transmission period (n_i), for i=1 to 60, was calculated as:

$$n_i = \delta \times N$$

Given the number of bulls becoming infected each of the days during the transmission period (n_1 to n_{60}) and the limit (in days) for the first level of test sensitivity depending on the time since the infection of the animal (d_1), the number of bulls within this level of test sensitivity at time t_{c1}, i.e., when the first control was performed (n_{c1d1}) was calculated as:

$$n_{c_1 d_1} = \sum_{k=1}^{d_1} n_k \qquad \text{(For } k < t_{c1}\text{)}$$

Similarly, the number of bulls within the last level of test sensitivity (d_y) at time t_{c1}, i.e., when the first control was performed (n_{c1dy}) was calculated as:

$$n_{c_1 d_y} = \sum_{k=d_{y-1}}^{d_y} n_k \qquad \text{(For } k < t_{c1}\text{)}$$

Likewise, the numbers of bulls within each level of test sensitivity, at the times when the remaining controls were performed (n_{c2d1} to n_{cxdy}), were also calculated.

For a bull included within level d_1 (infected for less than d_1 days), the probability that the diagnostic test correctly identifies the infection status was given by Se_{d1}. Therefore, at the time of the first control (t_{c1}), the probability that BTV infection was identified in any of the n_{c1d1} bulls infected for less than d_1 days (Se_{c1d1}) was calculated as:

$$Se_{c_1 d_1} = 1 - \left(1 - Se_{d_1}\right)^{n_{c_1 d_1}}$$

The overall probability that BTV infection of the SCC was identified if bulls were tested at the time of the first control (Se_{c1}), was calculated as:

$$Se_{c_1} = 1 - \prod_{r=1}^{y} \left(1 - Se_{c_1 dr}\right)$$

Similarly, the overall probabilities that BTV infection of the SCC was identified if bulls were tested at the time of the remaining controls (Se_{c2},… Se_{cx}), were also calculated.

The probability of BTV detection at the first control (P_{Dc1}) was estimated by multiplying the probability that the animals were tested at time t_{c1} (P_{c1}) by the overall Se at time t_{c1} (Se_{c1}):

$$P_{Dc_1} = P_{c_1} \times Se_{c_1}$$

The overall probability of detection given *x* controls (P_D) may be estimated as:

$$P_D = 1 - \prod_{s=1}^{x} (1 - P_{Dcs})$$

II- Non-simultaneous testing

When non-simultaneous controls were assumed, the probabilities that the animals were controlled at times t_{c1},.., t_{cx} (P_{c1},.., P_{cx}) were specific for each animal. Consequently, the probability of detection (P_D) was estimated independently for each animal, and later combined to obtain the overall probability of detection. Given *N* animals present in the SCC, the overall probability of BTV detection was calculated as:

$$P_D = 1 - \prod_{k=1}^{N} (1 - P_{Dk})$$

III- Testing of semen samples

If testing was carried out on semen samples, the estimation of the probability of BTV transmission by semen would be similar to that of non-simultaneous testing, but in this case a single test, at the time of the semen collection would be performed.

As there is no information on test sensitivity for BTV detection in bovine semen (Wentink et al. 2000), a sensitivity equivalent to that of PCR in serum samples was assumed.

3.3.2.1.1.4. <u>Probability of BTV shedding in semen</u>

Studies carried out in the 1970s (Luedke et al. 1977; Breckon et al. 1980) suggested that some bulls may intermittently excrete virus in their semen, which led to restrictions in the international trade of semen. However, further attempts to confirm these theories were not successful (Wrathall et al. 2006). Studies with several serotypes (BTV-1, BTV-3, BTV-16, BTV-20, and BTV-21) carried out by different authors (Gard et al. 1989; Kirkland et al 2004) failed to isolate BTV from a total of 231 semen samples from viraemic bulls.

However, in a recent study (Vanbinst et al. 2010), out of 89 extended semen samples from viraemic bulls, 48 were positive to BTV-8 by a duplex real-time RT-PCR, and 30 were doubtful. Furthermore, the presence of live, virulent BTV was demonstrated by the isolation of the virus in 4 out of 9 of the positive samples.

Given this marked difference, two different groups of BTV serotypes were considered: the low-risk serotypes and BTV-8. The probability of BTV transmission was estimated independently for these two groups.

Probability calculations

For the low risk serotypes, based on the failure to isolate BTV from 231 semen samples from viraemic bulls (Gard et al. 1989; Kirkland et al 2004), the probability of virus shedding in semen of viraemic bulls was represented by a *Beta* distribution in which the number of trials was 231, and the number of successes was 0 (OIE 2004b).

For BTV-8, as the detection of BTV by real-time RT-PCR does not necessarily mean presence of live virus, the probability of virus shedding in semen was calculated as the product of:

a) Probability of BTV detection by real-time RT-PCR, modelled by a *Beta* distribution (OIE 2004b), in which the number of trials was 89, and the number of successes was represented by a *Uniform (48,78)* distribution to account for the uncertainty in relation to the doubtful results.

b) Probability of BTV isolation from PCR-positive samples, modelled by a *Beta (4, 6)* distribution

3.3.2.1.1.5. Probability of BTV not being detected at the SCC (after semen collection)

The animal health requirements applicable to intra-Community trade of bovine semen are laid down in Council Directive 2003/43/EC (Anonymous 2003). Annex C (3)(a) states that frozen semen for intra-Community trade must be stored in approved conditions for a minimum period of 30 days prior to dispatch. That means that if, within this 30 days period, BTV infection was detected in any of the animals in the SCC, it was assumed that no movement of semen from that SCC would be allowed. In order to assess the effectiveness of this 30 days delay in preventing the risk of BTV transmission by semen, the probability of BTV detection post-collection was estimated. This probability was estimated as in before collection, but in this case the controls were performed between the day on which the semen sample was collected (*C*) and the following 30 days.

3.3.2.1.1.6. Probability that BTV-infected semen infects recipient cows

Even though bulls were reportedly able not only to excrete BTV in semen, but also to infect cows at natural mating (Luedke et al. 1977; Breckon et al. 1980), further experiments (Parsonson et al. 1994) failed to reproduce these findings. However, seminal shedding of BTV was achieved using experimental infection (Bowen & Howard 1984), and frozen-thawed extended semen from these bulls was used to inseminate 9 heifers, 3 of which became viraemic. Therefore, the probability of infection of recipient cows by BTV infected semen was represented by a *Beta (4, 7)* distribution.

Finally, for each month after the infection of the SCC (*m*), the probability that a single semen sample collected resulted in BTV transmission (P_{Tm}) was calculated as the product of the probabilities of the six different steps (figure 1).

3.3.2.1.2. Estimation of the annual probability of BTV transmission by semen (P_T)

To account for the fact that BTV transmission is restricted to a limited period of the year, the annual probability of BTV transmission by semen for a single semen dose (P_T) was calculated.

For P_{Tm} being the probability that a single semen dose obtained from semen collected within month m after the infection of the SCC resulted in BTV transmission, and P_m being the proportion of doses obtained from semen collected in month m, the mean annual probability of BTV transmission by a single semen sample (P_T) was calculated as:

$$P_T = \sum_{m=1}^{12} \left(P_{Tm} \times P_m \right)$$

Collection of semen samples was assumed to be homogenously distributed throughout the year, and therefore P_m was equal to 1/12.

3.3.2.2. Sensitivity analysis

In order to identify those inputs which were more influential on the final output (probability of BTV transmission by semen), a sensitivity analysis was carried out using the rank order correlation method, which is based on the Spearman rank correlation coefficient calculations. With this analysis, the rank correlation coefficient is calculated between the selected output variable and the samples for each of the input distributions. The higher the correlation between the input and the output, the more significant the input is in determining the value of the output. The rank order correlation is the method recommended by the OIE, as no assumptions are made about the nature of the relationship between the variables (OIE 2004b).

3.3.2.3. Software

The spreadsheet model was constructed in Microsoft Excel (Microsoft® Office 2007, Redmond, WA). The model was run for 20,000 iterations (Latin Hypercube sampling) in @Risk version 5.5.0 (© Palisade Corporation, Ithaca, NY). This allowed the convergence of all the output probability distributions. The sensitivity analysis was performed using @Risk version 5.5.0.

3.3.3. Results

Results for low-risk serotypes and BTV-8 are presented in tables 2 & 3, respectively. They only differ in the probability of BTV shedding in semen, and therefore probabilities of BTV transmission by semen in both groups are proportional, while the probabilities for the remaining steps are the same.

With the exception of when ELISA controls and simultaneous testing were applied, a low within-farm spread resulted in an increased risk of BTV transmission by semen of between 6.7 times and $2.4x10^6$ times in comparison with a high within-farm spread (tables 2 & 3).

For most of the scenarios considered, the majority of the risk (between 57% and almost 100%) was derived from animals in which the semen sample was collected within the first month after infection of the SCC (month 1), while after month 2 the risk of BTV transmission by semen was virtually negligible. The exception was the ELISA/Simultaneous/Low scenario, in which 53% of the risk was due to animals in which the semen sample was collected between the first and second month after infection of the SCC (month 2), and 4% of the risk was extended to month 3. Therefore, results are only reported for month 1 to month 4 (tables 2 & 3).

MEAN PROBABILITIES	ELISA Sim. Low	ELISA Sim. High	ELISA Non-Sim. Low	ELISA Non-Sim. High	PCR Sim. Low	PCR Sim. High	PCR Non-Sim. Low	PCR Non-Sim High	Semen Low	Semen High	No controls Low	No controls High
Infected and viraemic												
Month 1	1.01E-02	5.10E-02	1.04E-02	5.06E-02	1.02E-02	5.10E-02	1.00E-02	5.02E-02	1.01E-02	5.08E-02	1.01E-02	5.04E-02
Month 2	3.30E-02	1.66E-01	3.24E-02	1.65E-01	3.31E-02	1.64E-01	3.28E-02	1.63E-01	3.29E-02	1.66E-01	3.30E-02	1.66E-01
Month 3	2.79E-02	1.38E-01	2.76E-02	1.38E-01	2.77E-02	1.39E-01	2.76E-02	1.39E-01	2.79E-02	1.40E-01	2.80E-02	1.39E-01
Month 4	2.01E-02	9.90E-02	1.95E-02	9.81E-02	1.98E-02	1.00E-01	1.95E-02	9.98E-02	1.99E-02	9.79E-02	1.96E-02	9.76E-02
Detection Pre-collection[†]												
Month 1	2.66E-02	7.50E-02	3.38E-02	1.35E-01	2.79E-01	3.84E-01	3.71E-01	6.36E-01	6.13E-01[‡]	7.60E-01[‡]	NA	NA
Month 2	3.39E-01	5.12E-01	4.63E-01	8.87E-01	9.52E-01	9.95E-01	9.80E-01	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Month 3	7.91E-01	9.40E-01	9.11E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Month 4	9.82E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Controlled Pre-collection[†]												
Month 1	2.46E-01	2.51E-01	9.06E-01	9.03E-01	5.35E-01	5.32E-01	9.57E-01	9.55E-01	9.90E-01[‡]	9.89E-01[‡]	NA	NA
Month 2	7.51E-01	7.49E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Month 3	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Month 4	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00[‡]	1.00E+00[‡]	NA	NA
Detection Post-collection[†]												
Month 1	3.10E-01	4.38E-01	4.50E-01	8.84E-01	9.51E-01	9.95E-01	9.79E-01	1.00E+00	9.95E-01[‡]	9.95E-01[‡]	NA	NA
Month 2	4.80E-01	5.01E-01	8.57E-01	9.99E-01	1	1.00E+00	1.00E+00	1.00E+00	9.95E-01[‡]	9.95E-01[‡]	NA	NA
Month 3	5.00E-01	5.00E-01	1.00E+00	1	1	1.00E+00	1.00E+00	1.00E+00	9.95E-01[‡]	9.95E-01[‡]	NA	NA
Month 4	5.03E-01	5.01E-01	1.00E+00	1	1	1.00E+00	1.00E+00	1.00E+00	9.95E-01[‡]	9.95E-01[‡]	NA	NA
Controlled Post-collection[†]												
Month 1	5.03E-01	4.99E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	NA	NA	NA	NA
Month 2	4.98E-01	5.01E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	NA	NA	NA	NA
Month 3	5.01E-01	5.00E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	NA	NA	NA	NA
Month 4	5.03E-01	5.01E-01	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	NA	NA	NA	NA
Monthly probabilities of BTV transmission by semen (P_{Tm})												
Month 1	8.47E-06	2.80E-05	5.91E-06	1.47E-06	9.27E-08	4.87E-09	2.09E-08	8.74E-15	4.05E-09	4.53E-10	1.59E-05	7.91E-05
Month 2	1.06E-05	1.48E-05	4.26E-06	5.05E-08	2.48E-09	8.67E-13	2.01E-10	3.05E-26	2.54E-12	2.71E-26	5.21E-05	2.61E-04
Month 3	8.32E-07	1.26E-09	3.71E-09	1.75E-15	4.26E-14	2.28E-24	2.94E-15	0.00E+00	4.46E-15	1.12E-44	4.25E-05	2.17E-04
Month 4	1.73E-08	1.41E-15	0.00E+00	0.00E+00	4.24E-19	0.00E+00	2.38E-20	0.00E+00	3.23E-15	7.01E-45	3.06E-05	1.51E-04
Annual probability of BTV transmission by semen (P_T)	1.66E-06	3.57E-06	8.48E-07	1.27E-07	7.93E-09	4.06E-10	1.76E-09	7.20E-16	3.38E-10	3.78E-11	1.18E-05	5.91E-05

Table 2: Low-risk serotypes: A) Mean probabilities (per semen dose) for different steps and months after infection of the SCC, in the different scenarios considered. B) Mean annual probability of BTV transmission (per semen dose) in the different scenarios considered.

[†] *To help the interpretation, the results are expressed as probabilities of detection and control (pre and postcollection) instead of probabilities of no-detection and no-control*

[‡] *In semen scenarios detection and control postcollection is by testing of the semen sample whose risk is being assessed, and detection and control precollection is by testing of the semen samples from other animals.*

Sim.: Simultaneous, Non-sim.: Non-simultaneous, NA: Not applicable

MEAN PROBABILITIES	ELISA Sim. Low	ELISA Sim. High	ELISA Non-Sim. Low	ELISA Non-Sim. High	PCR Sim. Low	PCR Sim. High	PCR Non-Sim. Low	PCR Non-Sim High	Semen Low	Semen High	No controls Low	No controls High
Monthly prob. of BTV transmission by semen (P_{Tm})												
Month 1	6.28E-04	2.06E-03	4.28E-04	1.08E-04	6.56E-06	3.56E-07	1.52E-06	7.92E-13	2.84E-07	4.02E-08	6.28E-04	2.06E-03
Month 2	7.52E-04	1.12E-03	3.24E-04	3.42E-06	2.02E-07	8.86E-11	1.47E-08	1.79E-24	1.86E-10	3.45E-24	7.52E-04	1.12E-03
Month 3	6.29E-05	1.06E-07	1.96E-07	3.50E-12	3.49E-12	1.10E+22	2.29E-13	0.00E+00	3.26E-13	7.82E-43	6.29E-05	1.06E-07
Month 4	1.06E-06	6.06E-14	0.00E+00	0.00E+00	3.09E-17	0.00E+00	1.77E-18	0.00E+00	2.29E-13	6.25E-43	1.06E-06	6.06E-14
Annual prob. of BTV transmission by semen (P_T)	1.20E-04	2.65E-04	6.26E-05	9.29E-06	5.64E-07	2.97E-08	1.29E-07	6.60E-14	2.37E-08	3.35E-09	1.20E-04	2.65E-04

Table 3: BTV-8: A) Mean probabilities (per semen dose) for different months after infection of the SCC, in the different scenarios considered. B) Mean annual probability of BTV transmission (per semen dose) in the different scenarios considered.

† *To help the interpretation, the results are expressed as probabilities of detection and control (pre and postcollection) instead of probabilities of no-detection and no-control*

‡ *In semen scenarios detection and control postcollection is by testing of the semen sample whose risk is being assessed, and detection and control precollection is by testing of the semen samples from other animals.*

Sim.: Simultaneous, Non-sim.: Non-simultaneous, NA: Not applicable

The probability of detection post-collection was between 1.6 and 13.3 times higher than pre-collection for month 1, but this difference decreased progressively in the following months (and even when ELISA/simultaneous tests were performed, after month 3, detection pre-collection was more likely than post-collection).

Non-simultaneous controls would allow increasing the probability that the animals were controlled and therefore detected by between 2 and $5.6x10^5$ times.

When compared with the scenario in which no controls were carried out, ELISA controls allowed reducing the risk by between 7 and $4.7x10^2$ times (depending on the rate of spread and the timing of controls), while PCR controls allowed reducing the risk by between $1.5x10^3$ and $8.2x10^{10}$ times (depending on the rate of spread and the timing of controls). If controls were performed on each semen sample, the risk reduction achieved would vary between $3.5x10^4$ and $1.6x10^6$ times for low and high rate of spread respectively.

Sensitivity analysis

The most influential input parameters on the risk of BTV transmission by semen were (table 4): the day semen sample was collected (*C*) within months 1 & 2, the probability of BTV shedding in semen of viraemic bulls, the duration of viraemia (*V*) for month 2, the latent period (*LP*) for month 1 and the probability that semen infects a recipient cow. However, important differences existed among the scenarios considered.

MEAN PROBABILITIES	ELISA Sim. Low	ELISA Sim. High	ELISA Non-Sim. Low	ELISA Non-Sim. High	PCR Sim. Low	PCR Sim. High	PCR Non-Sim. Low	PCR Non-Sim High	Semen Low	Semen High	No controls Low	No controls High
Day semen sample collected (C)/Month 1	0.25	0.29	0.29	0.34	0.33	0.07	0.32	-0.04	0.35	0.05	0.12	0.12
Day semen sample collected (C)/Month 2	-0.13	-0.18	-0.25	-0.24	-0.18	-0.01	-0.18	0.00	-0.18	0.00	-0.02	-0.04
Probability of BTV shedding in semen of viraemic bulls	0.26	0.09	0.25	0.08	0.10	0.05	0.21	0.05	0.20	0.03	0.51	0.49
Duration of viraemia (V)/Month 2	0.18	0.15	0.15	0.13	0.15	0.00	0.16	0.00	0.06	0.01	0.14	0.15
Latent period (LP)/Month 1	-0.07	-0.10	-0.10	-0.12	-0.11	-0.10	-0.11	-0.10	-0.13	-0.15	-0.03	-0.04
Probability semen infects recipient cow	0.09	0.03	0.08	0.04	0.04	0.03	0.06	0.01	0.08	0.02	0.19	0.18

Table 4: Results of the sensitivity analyses for the different scenarios considered

3.3.4. Discussion

The model showed that an increase in the rate of spread within the SCC resulted in an increased probability of infection and viraemia, but also in an even higher increase in the probability of detection (mainly pre-collection), which reduced the risk of BTV transmission by semen. The exception was when simultaneous ELISA controls were performed, as the increase in detection was lower than the increase in probability of infection and viraemia.

In general, even though the probability of infection and viraemia increased from month 1 to month 2, the probability of detection increased even more, and as a result the highest risk of BTV transmission was when the semen sample was collected within the 30 days after infection (month 1). After month 2, it was unlikely for infected animals not to be detected, and therefore the risk was further reduced. However, in the ELISA/Simultaneous/Low scenario, the slower increase in the probability of detection delayed the highest risk to month 2.

The storage of semen for 30 days prior to dispatch seemed to be an efficient way of reducing the risk of transmission by semen because the probability of detection after collection was much higher than before collection in month 1, which was responsible for the majority of the risk.

Non-simultaneous testing of the animals allowed a reduction of the risk of BTV transmission by semen (as compared to simultaneous testing) by increasing the probability that at least one of the bulls in the SCC were tested. However, this reduction was limited in the case of ELISA tests and low rate of spread (reduction by 2 times), while the reduction was $5.6x10^5$ times in the case of PCR tests and high rate of spread.

Serological controls of donor animals every 60 days was not a very effective method for preventing the risk of BTV transmission by semen, because frequently the potentially infectious semen may be sent out of the SCC despite the donor bull not having been subjected to any control. This lack of efficiency was exacerbated when semen was collected within month 1 because the sensitivity of the test for recently infected animals was very low. In contrast, PCR controls of donor animals every 28 days seemed to be much more effective because a) the probability of donor animals being subjected to controls was much higher, and b) the sensitivity of the test was quite high even for animals infected for just 3 days.

If tests were carried out on semen samples, the high sensitivity of the PCR implied that the probability of an infected semen sample yielding a negative result (false negative) was low. Besides, for a semen dose obtained from that sample to be sent out of the SCC, all previously tested samples in that SCC would need to have yielded negative results. Because of that, after month 1, the probability that testing of semen samples failed to detect BTV infection at the SCC was very low. These results were based on the assumption that sensitivity of the PCR in

semen was equivalent to that in serum samples. Diagnostic tests on semen may be affected by virucidal properties, cell culture cytotoxicity and inhibition of reverse transcriptase enzyme of seminal plasma of raw semen However, dilution with semen extenders and use of PCR may help to improve the sensitivity (MacLachlan & Osburn 2006). The real-time RT-PCR was reported to be a sensitive method for the detection of BTV in extended semen samples (Vanbinst et al. 2010), although only analytical sensitivity (based on probit analysis) was measured.

The sensitivity analysis showed that, in general, the day the semen sample was collected (*C*) within month 1 was the most influential parameter, and there was a positive correlation between collection day and probability of BTV transmission by semen, so that the later the sample was collected within month 1, the longer the animal had to get infected and overcome the latent period, and the higher the risk of BTV transmission by semen. In contrast, there was a negative correlation with the day the semen sample was collected within month 2, so that the sooner the semen sample was collected within month 2, the higher the risk of BTV transmission by semen. The probability of BTV shedding in semen was also identified as a crucial parameter in the probability of BTV transmission by semen, in particular for scenarios in which BTV spread was low and when control measures were less effective or not applied. In contrast to the day of semen collection within month 1 and month 2, which were variable parameters, there is a great degree of uncertainty associated with the probability of BTV shedding in semen. The assumption that bulls may shed BTV in their semen is derived from studies carried out in the 1970s (Luedke et al. 1977; Breckon et al. 1980), and that led to constraints on the international trade of semen. However, further attempts were not able to confirm this theory and this failure was attributed to the intermittent excretion of BTV in semen. The possibility of virus shedding seemed to be related to the type of virus ("wild type" *vs.* laboratory-adapted) and to the age of the animals (Kirkland & Hawkes 2004): BTV was often detected in semen of old bulls infected with laboratory-adapted viruses, and in semen of some old bulls infected with "wild" strains, although it was believed that the virus was present in semen as a result of inflammation or because of the presence of blood in semen. The uncertainties regarding the epidemiology of BTV were used to justify protectionist trade barriers imposed by some BTV-free countries with severe economic consequences (MacLachlan & Osburn 2006). However, the presence of live BTV was recently confirmed in 54% of the semen samples from bulls naturally infected with BTV-8, by a combination of PCR and virus isolation (Vanbinst et al. 2010). The fact that virulent wild-type BTV-8 is shed easily in semen indicates that there are important differences in the probability of BTV shedding in semen depending on the serotype.

Duration of viraemia was also identified as an influential parameter in the sensitivity analysis, while the latent period (*LP*) for month 1 and the probability that semen infects recipient cows seemed important only for some scenarios. Even though previous experiments with semen potentially infected with BTV failed to produce infection of recipient cows (Parsonson et al. 2010), Bowen and collaborators (1985) were able to infect 3 out of 9 heifers using semen from experimentally infected bulls. BTV-8 seems to share characteristics with laboratory-adapted viruses, such as the ability to cross the placenta and cause fetal infections, and that makes it more likely to be transmitted venereally than other BTV serotypes (Biosecurity Australia 2010).

Model calculations were based on the assumption of a 2-month transmission period, even though for some countries this period may be longer. However, a longer transmission period would only affect the probabilities of infection from month 3 onwards, and after month 2, the probability of no detection of BTV at the SCC was so low that it would not significantly influence the results.

In order to explore the effect of the number of donor bulls within a SCC on the risk of BTV transmission by semen, the model was also run assuming 10 animals at the SCC (results not shown). A reduction in the number of animals in the SCC resulted in a decrease of the probability of BTV detection at the centre, and therefore an increase of the probability of a semen dose transmitting BTV. This increase ranged from 1.3 times for the ELISA/Simultaneous/High scenario and $3.2x10^4$ times for the PCR/Non-simultaneous/High scenario.

BTV infection in cattle is almost always subclinical (Elbers et al. 2008b) and therefore the possibility of clinical detection of infected bulls was not taken into account. However, as with BTV-8, sporadic cases of clinical disease in cattle may occur, and that would result in an increase of the probability of detection and therefore a reduction of the risk of BTV transmission by semen.

Fresh semen is not required to be stored for 30 days prior to dispatch, and therefore poses a higher risk than with frozen semen.

The model is based on the assumption that the SCC was already infected. Moreover, for the confirmation of a BTV outbreak in a previously free area, the virus needs to have circulated in the area, and that depends on the presence of favorable conditions for BTV transmission. Therefore, the actual probability of BTV introduction in a previously free area as a consequence of the importation of one semen dose (results are calculated per semen dose) would be much lower. On the other hand, for the calculation of the overall probability of BTV introduction (by semen) in this free area, the total number of semen doses imported would have to be taken into account.

The model provides a framework for the estimation of the risk by other pathogens transmitted by semen and the assessment of the preventive measures applied. There is a great deal of uncertainty in relation to two important parameters in the risk of BTV transmission: the probability of BTV shedding in semen and the probability BTV-infection of recipient cows. The clarification of this uncertainty would be crucial to determine whether preventive measures are effective in reducing the risk to negligible levels.

4. DISCUSSION

Besides Cyprus, where BT occurred regularly, only two BTV epidemics affected Europe before 1998. Between 1998 and 2005, a substantial change in the epidemiology of BT occurred and several BTV serotypes (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16) were introduced into Southern and Eastern Europe. The origins of these introductions were attributed to either animal movement or transportation of infected *Culicoides* on the wind.

Before 2006, BTV was widely perceived in northern Europe as an exotic virus that had a low probability of introduction into, spread through and persistence in the region (Carpenter et al. 2009a). However, in August 2006, BTV-8 was detected in the Netherlands, from where it spread to most of the country and to Belgium, Germany, France and Luxembourg (Wilson & Mellor 2009) causing the infection of over 2000 holdings. In the Netherlands alone, where 456 farms were affected (EUBTNET), the financial consequences of the BTV-8 epidemic in 2006 were estimated in 32 million Euros (Velthuis et al. 2010). In this case, the investigation on the possible routes of introduction of BTV-8 revealed that the most obvious mechanisms for BTV incursion into a free area, the importation of infected hosts or the transportation of infected *Culicoides* on airstreams seemed unlikely (Mintiens et al. 2008a). In order to estimate the potential for future introductions of BTV an understanding of the importance of the potential routes of virus introduction would be crucial (Carpenter et al. 2009a)

Given that BTV-8 totally bypassed Southern Europe, the introduction via other mechanisms, specifically, the potential for *Culicoides* to be imported along with or independently of the import of animals, plants or other 'materials', was also assessed (Mintiens et al. 2008a). There are several documented examples of transportation of insects and their pathogens via transport and trade networks: introduction of *Aedes aegypti* and yellow fever into America from Western Africa; introduction of *Anopheles gambiae*, the most efficient vector of *Plasmodium falciparum* malaria, into Brazil also from Western Africa; or the most recent introduction of *Aedes albopictus*, vector of for example West Nile virus, into Europe and America from the Pacific islands (Lounibos 2002). However, *Culicoides* because of its small size, fragile nature and specialist taxonomy have been mainly ignored, and therefore the data available is scant (Carpenter et al. 2009a). Reye (Reye 1964) reported the possible spread of *Culicoides* by aircraft from Fiji to the Society Islands. Nie and collaborators (Nie et al. 2005) found *Culicoides* in 9 out of the 70 ships inspected at Qinhuangdao port, China. Cagienard and collaborators (2006b) considered the introduction via airplanes as one of the potential explanations for the finding of a *Culicoides imicola* specimen near the airport of Lugano.

The aim of **study II** was to assess, by means of a stochastic risk assessment model, the probability of development of a BTV outbreak as a consequence of the introduction of infected *Culicoides* via transport and trade networks. The model was applied to calculate the risk of a

BTV-8 epidemic in Spain in 2007 as the consequence of the transport of *Culicoides* from the affected Northern European countries (Germany, Belgium, the Netherlands, France, the Czech Republic, Denmark and the UK), regardless of the mechanism by which the midge was introduced.

The results indicate that the weighted annual risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of a single infected *Culicoides* from the affected Northern European countries seemed to be low (between $3.2x10^{-7}$ and $6.4x10^{-12}$), although there were major differences among countries. The highest risks were by *Culicoides* transported from Belgium, the Netherlands, Germany and France, which, as there were no significant differences in temperature among countries, was mainly a consequence of the fact that these countries had the highest proportion of affected farms, while the risk by *Culicoides* transported from Denmark, the UK, Switzerland and the Czech Republic was much lower because the proportion of farms affected in those countries was low.

The low risk is consistent with the known fact that the probability of an individual *Culicoides* being able to transmit BTV is low as a consequence of the low probabilities that a *Culicoides* will: a) feed on a viraemic host, b) be competent to transmit the virus, c) survive the *EIP* and d) subsequently feed on a susceptible host (Wittmann & Baylis 2000). Within a country affected by a BTV epidemic, only a proportion of farms are infected by BTV, within an affected farm, only a proportion of animals are infected by BTV, and these infected animals are infectious only during the viraemic period. In addition, not all female midges within a vector species are susceptible to infection with BTV or if infected are competent to transmit the virus. A series of barriers exists within certain individuals which either prevent or restrict virus infection (Mellor 2000), and as a consequence, every population of a vector species of *Culicoides* has a variable proportion of these refractory individuals (Mellor 2000). BTV transmission is also hampered by the effect of temperature. Generally speaking, at higher temperatures a vector may blood-feed more frequently and the rate of virogenesis within a vector is usually faster, leading to an enhanced probability of transmission. On the contrary, increase in temperature may shorten the lifespan of the vector, which would lessen the transmission potential. As temperature decreases, virogenesis usually slows and at some point may cease altogether; however, at lower temperatures the lifespan of the vector may be extended. The likelihood of BTV transmission by *Culicoides* is therefore a function of the interaction of these two opposing trends (Mellor 2000).

The risk of a BTV-8 outbreak in Spain because of the transportation of *Culicoides* from the majority of countries peaked in October, while for *Culicoides* from Belgium peaked in September, and from the Czech Republic in November. These peaks were mainly a

consequence of the moment in which the incidence of BTV infection of farms in the country of origin reached the maximum value, but were also dependent on the temperatures in the country of origin and destination, which determined the time needed for the infection of the vector and the transmission to a susceptible host.

The results for the different steps in the transmission pathway (depending on the month of emergence of *Culicoides* in Belgium) indicated that the months in which BTV transmission was not possible this was due to the fact that vectors were not able to survive the *EIP* and the *TNBM*. This was the consequence of the restrictive conditions in relation to temperature required for virogenesis, as for the completion of the *EIP*, mean daily temperatures need to consistently reach values above 11^{o}C. For the months in which BTV transmission was possible, the probability of a *Culicoides* getting infected was the most determinant factor for the low overall probability obtained. This is the consequence of on the one hand the low monthly probabilities of the hosts being viraemic, and on the other the low proportion of bites on an infectious host that infect a midge.

The sensitivity analysis identified the longevities of *Culicoides* in the months in which BTV transmission was more intense as the most influential parameters in the risk of a BTV-8 outbreak in Spain in 2007. This is a reflection of the fact that the longevity of *Culicoides* influences the probabilities for the first 4 steps in the transmission pathway (probabilities of infection, survival of the *EIP*+*TNBM*, transport and finding of a susceptible host).

An initial difficulty in defining the risk of a BT incursion lies in assessing the frequency and mechanism of introduction of pathogens or pathogen-infected hosts into an area, together with their associated probability of onwards transmission. To date, the best-characterized mechanisms for BTV incursion are via the movement of viraemic hosts or animal products from affected areas or via dispersal of infected *Culicoides* on airstreams (Carpenter et al. 2009a). One largely unaddressed aspect of BT epidemiology has been the potential for movement of infected adults *Culicoides* via local and global transportation networks (Carpenter et al. 2009a), and therefore the risk had to be estimated per vector, i.e. given a *Culicoides* hatched in month *i* in country *c*, the probability of emergence of a BTV-8 epidemic in Spain as a result of the introduction of that vector (by any of the possible means), was calculated. For the calculation of the actual probability of a BTV outbreak in a given country, further research on the probability of *Culicoides* transportation via different transport and trade networks, or the effect of transport on vector survival would be needed. However, it is clear that given that the probabilities per vector are low, for this mechanism to pose a significant risk to BTV-free countries, the number of vectors transported would have to be huge.

The introduction of BTV into a free area is also considered that may occur through the movement of semen (Saegerman et al. 2008). In fact, this was another of the hypotheses for the introduction of BTV-8 into north-western Europe in 2006 (Mintiens et al. 2008a). In order to prevent transmission by semen of animals from a SCC located in endemic areas, while avoiding the ban on the movement of semen from this SCC, both article 2.2.13 of the OIE Terrestrial Animal Health Code and Annex III of Commission Regulation 1266/2007/EC contemplate different measures that may be applied.

The aims of **study III** were to assess, in case of introduction of BTV into a bovine SCC, both the risk of BTV transmission by semen and the risk reduction achieved by some of the preventive measures available. In order to achieve this, a stochastic risk assessment model was built. The model was applied to different scenarios, constructed according to: a) the type of diagnostic test and the interval between the controls of donor bulls (either ELISA every 60 days or PCR every 28 days), b) the rate of BTV spread within the SCC (either low or high), and c) the timing of tests (either simultaneous or non-simultaneous). Besides, the effectiveness of testing the semen samples was also assessed. The results were compared with the probability of transmission if no control measures were applied.

The results of the model indicated that, except in the case of simultaneous ELISA controls, an increase in the rate of spread within the SCC resulted in an increased probability of infection and viraemia, but also in an even higher increase in the probability of detection (mainly pre-collection), which reduced the risk of BTV transmission by semen.

In general, even though the probability of infection and viraemia increased from month 1 to month 2, the probability of detection increased even more, and as a result the highest risk of BTV transmission was when the semen sample was collected within the 30 days after infection (month 1). After month 2, it was unlikely for infected animals not to be detected, and therefore the risk was further reduced. However, in the ELISA/Simultaneous/Low scenario, the slower increase in the probability of detection delayed the highest risk to month 2.

The storage of semen for 30 days prior to dispatch seemed to be an effective method for the reduction of the risk of transmission by semen because the probability of detection before collection in month 1 (accountable for the majority of the risk) was very low.

Non-simultaneous testing of the animals allowed a reduction of the risk of BTV transmission by semen as compared to simultaneous testing, by increasing the probability that at least one of the bulls in the SCC were tested. However, this reduction was limited in the case of ELISA tests and low rate of spread (reduction by 2 times), while the reduction was of 5.6×10^5 times in the case of PCR tests and high rate of spread.

Serological controls of donor animals every 60 days was not a very effective method for preventing the risk of BTV transmission by semen, because frequently the potentially infectious semen may be sent out of the SCC despite the donor bull not having been subjected to any control. This lack of efficiency was exacerbated when semen was collected within month 1 because the sensitivity of the test for recently infected animals was very low. In contrast, PCR controls of donor animals every 28 days seemed to be much more effective because a) the probability of donor animals being subjected to controls was much higher, and b) the sensitivity of the test was quite high even for animals infected for just 3 days.

Assuming that sensitivity of the PCR in semen was equivalent to that in serum samples, PCR testing of semen samples would be an effective measure to reduce the risk of BTV transmission by semen.

A reduction in the number of animals in the SCC resulted in a decrease of the probability of BTV detection at the centre, and therefore an increase of the probability of a semen dose transmitting BTV. This increase ranged from 1.3 times for the ELISA/Simultaneous/High scenario and $3.2x10^4$ times for the PCR/Non-simultaneous/High scenario.

The sensitivity analysis showed that, in general, two variable parameters, the day the semen sample was collected within month 1, and to a lesser extent the day the semen sample was collected within month 2, were very influential, indicating that chance played an important role on the risk of BTV transmission by semen. The later the sample was collected within month 1 and the sooner the semen sample was collected within month 2, the higher the risk of BTV transmission by semen.

The probability of BTV shedding in semen was also identified as a crucial parameter in the probability of BTV transmission by semen, in particular for scenarios in which BTV spread was low and when control measures were less effective or not applied. In contrast to the day of semen collection within month 1 and month 2, which were variable parameters, there is a great degree of uncertainty associated with the probability of BTV shedding in semen. Studies carried out in the 1970s (Luedke et al. 1977; Breckon et al. 1980) suggested that some bulls may intermittently excrete virus in their semen, which led to restrictions in the international trade of semen. However, further attempts to confirm these theories were not successful (Wrathall et al. 2006), and studies with several serotypes carried out by different authors (Gard et al. 1989; Kirkland et al. 2004) failed to isolate BTV from semen of viraemic bulls. Nevertheless, the presence of live BTV was recently confirmed in 54% of the semen samples from bulls naturally infected with BTV-8, by a combination of PCR and virus isolation (Vanbinst et al. 2010), which indicates that there seem to be important differences in the probability of BTV shedding in semen depending on the serotype. The sensitivity analysis also identified

another uncertain parameter, probability that semen infects recipient cows as an important in some scenarios considered.

The clarification of the uncertainty associated to the probability of BTV shedding in semen and the probability of BTV-infection of recipient cows, would be critical to determine whether preventive measures are effective in reducing the risk to negligible levels.

The results of the model indicated that there was a great difference in the effectiveness of measures to prevent BTV transmission by semen depending on factors such as the type of diagnostic test and interval between the controls of donor animals, the rate of BTV spread within the SCC, the timing of controls or the number of animals in the SCC. Of the legal measures available to prevent BTV transmission by semen, PCR controls of donor animals proved to be much more effective than ELISA controls, not just because the sensitivity of the test is quite high even for animals infected for just 3 days, but also because of the shorter interval between the controls.

The capacity of BTV to survive the winter and reappear in the next season has been demonstrated in different areas of Southern and Eastern Europe and with different serotypes (Taylor & Mellor 1994; Calistri et al. 2004; Osmani et al. 2006). After the introduction of BTV-8 in Northern Europe in 2006, many hoped that the epidemic would be extinguished in the winter because temperatures in the area are considerably lower than the minimum temperature required for BTV transmission (Wilson & Mellor 2009). However, the virus was able to overwinter, and in 2007 the epidemic was far more extensive, and besides the countries affected on the previous year (the Netherlands, Belgium, Germany, France and Luxembourg) it expanded to Denmark, Switzerland, the Czech Republic and the UK, affecting nearly 60,000 holdings, and causing the most economically damaging bluetongue epidemic ever (Wilson et al. 2008). In the Netherlands alone, the net cost of the 2007 BTV-8 epidemic, which affected 5798 farms, was valued at 164-175 million Euros (Velthuis et al. 2010). The following year, BTV-8 was able to overwinter again, and even though a vaccination programme had been established over 27,000 holdings in Europe were infected, including countries not previously affected (Spain, Italy, Austria and Hungary).

A large number of mechanisms for BTV overwintering have been proposed, but to date their relative importance remain unclear. As long as the understanding of the overwintering mechanisms in the field remains poor, the continuous reappearance of a BTV serotype in the areas previously affected seems inevitable (Wilson et al. 2008).

The objective of **study I** was to assess the probability of BTV overwintering by horizontal transmission by persistence of the virus in either adult vectors (pathway *I*), ruminants (through

prolonged viraemia) (pathway *II*) or a combination of both (pathway *III*), by means of a stochastic risk assessment model. Besides, the model allowed assessing the role that the few *Culicoides* present during the *PLVA* and those which live inside buildings play on the probability of overwintering. The model was applied to a real scenario: overwintering in Germany between 2006 and 2007.

The results of the model indicate that, in Germany, between 2006 and 2007, the length of the *PLVA* (4 months) did not allow overwintering by midges emerged before this period (pathways *Ia* and *IIIa*) neither with the exophilic nor with the endophilic behaviour. This long *PLVA* did not allow overwintering by hosts infected before the *PLVA* (pathway *II*) either.

For exophilic *Culicoides*, overwintering was only possible by pathway *Ib* as temperatures above the virogenesis rate limit were reached only a few days in April, which did not allow the completion of the *EIP* and *TNBM*, and transmission to the host before the end of the *PLVA* (pathway *IIIb*). Endophilic behaviour appeared to favour overwintering mainly by increasing the probability by pathway *Ib*, and to a lesser extent by allowing the transmission of BTV to ruminants during the *PLVA* (pathway *IIIb*), which allowed advancing the period in which transmission was possible (to January).

Overall, the sensitivity analysis highlighted the importance of the temperature-dependent parameters (longevity, *EIP* and *TNBM*) on the probability of BTV overwintering, although their relative importance is difficult to assess because of the correlation that exists among these parameters. The importance of longevity may be understood because of its influence in both the probability of infection and the probability of surviving the *EIP* and the *TNBM*. On the other hand, the duration of the *TNBM* seemed to have a less decisive role in the probability of overwintering, which might be explained by the fact that when temperatures were favourable for the completion of the *EIP*, they also allowed the rapid completion of the *TNBM*. Of the non temperature-dependent parameters, the proportion of bites on an infectious host that infect a midge seemed to be the most influential. There is a great degree of uncertainty regarding this parameter as the distribution used was a combination of field estimates *C. sonorensis* and laboratory estimates for *C. obsoletus*, and variations in viral titres within the host and among different hosts, were not taken into account. The results of the sensitivity analysis are in agreement with previous studies (Gubbins et al. 2008), and emphasize the need for further research in the estimation of these influential parameters.

Even though endophily seemed to favour overwintering, its effect was limited (the mean weighted probabilities were less than 3 times higher than for exophilic *Culicoides*). This is a consequence of the complex effect of temperature on BTV transmission: an increase of temperature reduces the duration of the *EIP* and the *TNBM*, but also the longevity of

Culicoides; and a decrease of temperature increases the longevity of *Culicoides*, but also the duration of the *EIP* and the *TNBM*. Therefore, even though endophily (milder temperatures) increased the probability of vector infection, this probability is the result of the equilibrium between longevity and number of blood meals, and while endophily increased the number of blood meals in relation to exophily (lower temperatures), it also decreased longevity. Similarly, endophily increased the probability of surviving the *EIP* and the *TNBM*, but again, this probability is the result of the equilibrium between longevity and duration of the *EIP* and the *TNBM*, and while endophily decreased the duration of these 2 periods in relation to exophily, it also decreased longevity. This is somehow no unexpected because it is known that BTV transmission by *Culicoides* is inefficient, and that very few ever transmit the virus, so this has to be compensated by huge numbers of vectors (Wittmann & Baylis 2000).

Given the low probabilities obtained for the pathways considered in the model, for these mechanisms to have played a major role in overwintering in Germany, the number of vectors present in winter would have had to be large. Even though *Culicoides* captured represent only a fraction of the *Culicoides* population, the number of *Culicoides* trapped during winter in Germany seems too small (captures during the *PLVA* represent only a 0.06% of the total of the year). The low probabilities are consistent with what was observed in northern Europe, where the disease reappeared around areas of intense transmission rather than those where the transmission was most recent (EFSA 2007), and nearly all the northern European countries previously infected (Meiswinkel et al. 2008a). In fact, BTV isolation from overwintering populations of *Culicoides* has not been achieved yet (EFSA 2007).

Therefore, other overwintering mechanisms not considered in the model seem to have played a decisive role in overwintering in Germany. In 2008, transplacental transmission of field strains of BTV-8 was demonstrated in Northern Ireland (Menzies et al. 2008). Before this, it was thought only viruses passaged in tissue culture had the potential to cross the placenta, but since then, similar findings have been reported in several European countries (Darpel et al. 2009; De Clercq et al. 2008; Santman-Berends et al. 2010). In addition, live virus has also been isolated from some of these calves (Menzies et al. 2008). As the bovine gestation period is of 9 months duration, this mechanism should easily enable BTV-8 to overwinter, and therefore this would seem to be the most likely explanation for BTV-8 overwinter in Northern Europe (Mertens et al. 2008). However, given that live virus has been recovered only from a small proportion of PCR-positive calves, and that the infectivity of viraemic newborn calves to competent vectors is not yet known, the role of transplacental transmission in overwintering remains to be elucidated (Dal Pozzo et al. 2009).

Besides, mechanisms considered of minor significance during normal transmission, may become disproportionately important for the survival of the virus when normal transmission is interrupted by winter, and one or more of these mechanisms may be responsible for the cases of BTV transmission that have taken place during the winter in North-Western Europe (Wilson & Mellor 2009).

The model was applied to a given scenario, in this case Germany in 2006-2007 taking into account its specific conditions. Therefore, any conclusions drawn are specific of that scenario as different conditions (e.g. temperatures or duration of *PLVA*) may produce different results.

There is a consensus about the most important routes of BTV introduction but the importance of alternative, less frequent routes is not well understood. In this thesis we have build two models to calculate the risk by two of these routes and we have applied them for different scenarios. The risk in both cases seemed to be low, although not negligible. Similar results were obtained with the risk of overwintering in Germany by horizontal transmission by vectors, hosts or both. These models can be easily adapted to other diseases transmitted by *Culicoides* as African horse sickness or epizootic haemorrhagic disease.

5. CONCLUSIONS

Study I

Given the low probabilities obtained from the model and the limited number of vectors present in winter, overwintering in Germany between 2006 and 2007 seemed unlikely to have occurred by horizontal transmission in ruminants, in vectors or in both.

Overwintering was only possible by vectors infected during the period of low vector activity. If exophilic behaviour of *Culicoides* was assumed, transmission to the hosts occurred only after the period of low vector activity, while endophily allowed transmission both during and after this period, increasing the probability of overwintering, although the increase was only by less than 3 times.

Study II

The weighted annual risk of a BTV-8 outbreak in Spain in 2007 as the consequence of the transport of a single *Culicoides* from the affected Northern European countries seemed to be low (between 3.2×10^{-7} and 6.4×10^{-12}) although there were major differences depending on the country of origin, with the highest risks by *Culicoides* imported from Belgium, the Netherlands, Germany and France.

For the months in which BTV transmission was not possible this was due to the fact that temperatures did not allow to survive the extrinsic incubation period and the time to the next blood meal, while for the months in which BTV transmission was possible, the probability of a *Culicoides* getting infected was the most determinant factor for the low overall probability obtained.

Study III

Of the legal measures available to prevent BTV transmission by semen, PCR controls of donor animals every 28 days seemed to be between 2.1×10^{2} and 1.8×10^{8} times more effective than ELISA controls of donor animals every 60 days.

The storage of semen for 30 days prior to dispatch is an effective strategy for the reduction of the risk of transmission by semen.

The risk of BTV transmission by semen is influenced by several factors. In general, an increase in the rate of BTV spread, an increase in the number of donor bulls at the centre and the non-simultaneous testing of the animals increased the probability of detection and therefore decreased the probability of BTV transmission by semen.

6. SUMMARY

SUMMARY

Even though bluetongue virus (BTV) transmission is apparently interrupted during winter, bluetongue outbreaks often reappear in the next season (overwintering). Several mechanisms for BTV overwintering have been proposed, but to date, their relative importance remain unclear. In order to assess the probability of BTV overwintering by persistence in adult vectors, ruminants (through prolonged viraemia) or a combination of both, a quantitative risk assessment model was developed. Furthermore, the model allowed the role played by the residual number of vectors present during winter to be examined, and the effect of a proportion of *Culicoides* living inside buildings (endophilic behaviour) to be explored. The model was then applied to a real scenario: overwintering in Germany between 2006 and 2007. The results showed that the limited number of vectors active during winter seemed to allow the transmission of BTV during this period, and that while transmission was favoured by the endophilic behaviour of some *Culicoides*, its effect was limited. Even though transmission was possible, the likelihood of BTV overwintering by the mechanisms studied seemed too low to explain the observed re-emergence of the disease. Therefore, other overwintering mechanisms not considered in the model are likely to have played a significant role in BTV overwintering in Germany between 2006 and 2007.

Air, sea and land transport networks continue to expand in reach speed and volume, and one important consequences of this expansion is vector-borne pathogen importation. One important aspect of BT epidemiology which has not yet been addressed is the potential for movement of infected adults *Culicoides* via local and global transportation networks. Therefore, a stochastic risk assessment model was constructed to assess the probability of development of a BTV outbreak as a consequence of the introduction of infected *Culicoides* via transport and trade networks. The model was applied to calculate the risk of a BTV-8 epidemic in Spain in 2007 as the consequence of the transport of a *Culicoides* from the affected Northern European countries (Germany, Belgium, the Netherlands, France, the Czech Republic, Denmark and the UK), regardless of the mechanism by which the midge was introduced. The weighted annual risk by transportation of a single *Culicoides* from the affected Northern European countries seemed to be low (between 3.2×10^{-7} and 6.4×10^{-12}) although there were major differences among countries, with the highest risks by *Culicoides* imported from Belgium, the Netherlands, Germany and France. For this mechanism to pose a significant risk to BTV-free countries, large number of vectors would have to be transported.

Given that bluetongue (BT) may potentially be transmitted by semen, that the disease has significantly expanded in recent years, and that millions of doses of cattle semen are annually traded throughout the world, the transmission of bluetongue virus (BTV) by semen could have severe consequences in the cattle industry. The hypothesis that infected bulls could excrete BTV in their semen led to restrictions on international trade of ruminant semen and the establishment of measures to prevent BTV transmission by semen. However, neither the risk of BTV transmission by semen nor the effectiveness of these measures was estimated quantitatively. The objective of the study was to assess, in case of introduction of BTV into a bovine semen collection centre (SCC), both the risk of BTV transmission by bovine semen and the risk reduction achieved by some of the preventive measures available, by means of a stochastic risk assessment model. The model was applied to different scenarios, depending on for example the type of diagnostic test and the interval between the controls (testing) of donor bulls, or the rate of BTV spread within the SCC.

Enzyme-linked immunosorbant assay (ELISA) controls of donor bulls every 60 days seemed to be an ineffective method for reducing the risk of BTV transmission in contrast to polymerase chain reaction (PCR) tests every 28 days. An increase in the rate of spread within the SCC resulted in a reduced risk of BTV transmission by semen. The storage of semen for 30 days prior to dispatch seemed to be an efficient way of reducing the risk of transmission by semen.

The sensitivity analysis identified the probability of BTV shedding in semen as a crucial parameter in the probability of BTV transmission by semen. However, there is a great degree of uncertainty associated with this parameter, with significant differences depending on the BTV serotype.

RESUMEN

Aunque la transmisión del virus de la lengua azul (VLA) aparentemente se interrumpe durante el invierno, los brotes de lengua azul a menudo reaparecen en la siguiente temporada, es decir el virus es capaz de sobrevivir al invierno. Se han propuesto diferentes mecanismos para explicar este fenómeno, pero hasta la fecha, la importancia relativa de estos no está clara. Con el fin de evaluar la probabilidad de que el VLA persista tras el invierno a través de la persistencia en los vectores adultos, los hospedadores (por medio de una viremia prolongada) o una combinación de ambos, se ha desarrollado un modelo de evaluación del riesgo estocástico. Además, el modelo permite la evaluación por un lado del papel que juega el número residual de vectores presentes durante el invierno, y por otro el papel desempeñado por los *Culicoides* que tienen un comportamiento endofílico, es decir permanecen dentro de las granjas. El modelo se aplicó a un escenario real, en concreto la persistencia del VLA en Alemania entre 2006 y 2007. Los resultados mostraron que la presencia de vectores activos durante el invierno permitiría la transmisión de la lengua azul durante este periodo, y que mientras que la transmisión se vería favorecida por el comportamiento endofílico de algunos *Culicoides*, su efecto era limitado. A pesar de que la transmisión de la lengua azul por los mecanismos estudiados era posible, la probabilidad parecía demasiado baja para explicar la reaparición de la enfermedad que se observó en Alemania. Por tanto, otros mecanismos que no fueron considerados en este trabajo parecen haber jugado un papel más determinante en la supervivencia durante el invierno del VLA en dicho país entre 2006 y 2007.

Las redes de transporte aéreo, marítimo y terrestre continúan en expandiéndose, tanto en velocidad como en alcance y volumen, y una consecuencia importante de esta expansión es la importación de patógenos transmitidos por vectores. Un aspecto importante de la epidemiología de lengua azul, que aún no se ha logrado aclarar es la posibilidad de que *Culicoides* infectados sean introducidos a través de redes de transporte y comerciales. Por tanto, se desarrolló un modelo estocástico de evaluación de riesgos para calcular la probabilidad de aparición de un brote de lengua azul, como consecuencia de la introducción de *Culicoides* infectados a través de estas redes. El modelo se empleó para calcular el riesgo de que se produjera una epidemia del serotipo 8 del VLA en España en 2007 como consecuencia del transporte de *Culicoides* infectados desde los países afectados del norte de Europa (Alemania, Bélgica, Holanda, Francia, la República Checa, Dinamarca y el Reino Unido), independientemente del mecanismo por el cual se introdujo el vector. El riesgo anual ponderado como consecuencia del transporte de un *Culicoides* desde los países del norte de

Europa parecía ser baja (entre $3.2x10^{-7}$ y $6.4x10^{-12}$), aunque había grandes diferencias dependiendo del país de origen, con las probabilidades más elevadas debidas a la importación de vectores desde Bélgica, Holanda, Alemania y Francia. En cualquier caso, para que este mecanismo supusiera un riesgo significativo para los países libres de lengua azul, el número de vectores transportados tendría que ser muy elevado.

Dado que el VLA puede, potencialmente, ser transmitidos a través del semen, que la enfermedad se ha expandido significativamente en los últimos años, y que millones de dosis de semen de bovino se comercializan anualmente en todo el mundo, la transmisión del VLA por el semen podría tener consecuencias muy graves sobre la industria ganadera. La hipótesis de que los toros infectados pueden excretar virus a través de su esperma, dio lugar a restricciones en el comercio internacional de semen y al establecimiento de medidas para prevenir dicha transmisión. Sin embargo, ni el riesgo de transmisión del virus por el semen, ni la eficacia de estas medidas se han evaluado cuantitativamente. El objetivo del estudio fue calcular por medio de un modelo estocástico, en caso de introducción de la lengua azul en un centro de recogida de semen bovino (CRS), tanto el riesgo de transmisión del VLA a través de semen de bovino, como la reducción de dicho riesgo como consecuencia de la aplicación de las medidas preventivas disponibles. El modelo se aplicó a diferentes escenarios, dependiendo por ejemplo del tipo de prueba diagnóstica empleada, el intervalo entre los controles de los toros donantes, o la tasa de dispersión del VLA dentro del CRS. Los controles de los toros donantes cada 60 días por medio de la técnica de ensayo por inmunoabsorción ligado a enzimas (ELISA) parece ser un método poco eficaz para reducir el riesgo de transmisión del VLA por semen en comparación con el empleo de la reacción en cadena de polimerasa (PCR) cada 28 días. Un aumento en la tasa de propagación del VLA dentro del CRS resulta en una reducción del riesgo de transmisión del virus por el semen. El almacenamiento de semen por 30 días antes de su distribución parece ser una forma eficaz de reducir el riesgo de transmisión por el semen. El análisis de sensibilidad identificó la probabilidad de excreción del VLA en semen como un parámetro crucial en la probabilidad de transmisión por semen. Sin embargo, existe un alto grado de incertidumbre asociado a este parámetro, con diferencias significativas en función del serotipo del virus.

RESUM

Encara que la transmissió del virus de la llengua blava (VLlB) aparentment s'atura durant l'hivern, els brots de llengua blava sovint reapareixen en la següent temporada, és a dir, el virus és capaç de sobreviure a l'hivern. S'han proposat diferents mecanismes per explicar aquest fenomen, però fins ara la importància relativa d'aquests no està clara. Per tal d'avaluar la probabilitat que el VLlB persisteixi després de l'hivern a través de la persistència en els vectors adults, els hostes (per mitjà d'una virèmia prolongada) o una combinació d'ambdós, s'ha desenvolupat un model estocàstic d'avaluació del risc. A més, el model permet l'avaluació d'una banda del paper que juga el nombre residual de vectors presents durant l'hivern, i d'altra, el paper exercit pels Culicoides que tenen un comportament endofílic, és a dir romanen dins de les granges. El model es va aplicar a un escenari real, en concret la persistència del VLlB a Alemanya entre el 2006 i el 2007. Els resultats van mostrar que la presència de vectors actius durant l'hivern permetria la transmissió de la llengua blava durant aquest període, i que mentre que la transmissió es veuria afavorida pel comportament endofílic d'alguns Culicoides, el seu efecte era limitat. Tot i que la transmissió de la llengua blava pels mecanismes estudiats era possible, la probabilitat semblava massa baixa per explicar la reaparició de la malaltia que es va observar a Alemanya. Per tant, altres mecanismes que no van ser considerats en aquest treball semblen haver jugat un paper més determinant en la supervivència durant l'hivern del VLlB en aquest país entre el 2006 i el 2007.

Les xarxes de transport aeri, marítim i terrestre continuen en expansió, tant en velocitat com en abast i volum, i una conseqüència important d'aquesta expansió és la importació de patògens transmesos per vectors. Un aspecte important de l'epidemiologia de la llengua blava, que encara no s'ha aconseguit aclarir és la possibilitat que Culicoides infectats siguin introduïts a través de xarxes de transport i comercials. Per tant, es va desenvolupar un model estocàstic per avaluar el risc i calcular la probabilitat d'aparició d'un brot de llengua blava, com a conseqüència de la introducció de Culicoides infectats a través d'aquestes xarxes. El model es va emprar per calcular el risc que es produís una epidèmia del serotip 8 del VLlB a Espanya el 2007 com a conseqüència del transport de Culicoides infectats des dels països afectats del nord d'Europa (Alemanya, Bèlgica, Holanda, França, la República Txeca, Dinamarca i el Regne Unit), independentment del mecanisme pel qual es va introduir el vector. El risc anual ponderat com a conseqüència del transport d'un Culicoides des dels països del nord d'Europa semblava ser baixa (entre $3{,}2 \times 10^{-7}$ i $6{,}4 \times 10^{-12}$), encara que havia grans diferències depenent del país d'origen, amb les probabilitats més elevades degudes a la importació de vectors des de Bèlgica, Holanda, Alemanya i França. En qualsevol cas, perquè aquest mecanisme suposés un

risc significatiu per als països lliures de llengua blava, el nombre de vectors transportats hauria de ser molt elevat.

Atès que el VLlB pot, potencialment, ser transmès a través del semen, que la malaltia s'ha expandit significativament en els últims anys, i que milions de dosis de semen de boví es comercialitzen anualment a tot el món, la transmissió del VLlB pel semen podria tenir conseqüències molt greus sobre la indústria ramadera. La hipòtesi que els toros infectats poden excretar virus a través del seu esperma, va donar lloc a restriccions en el comerç internacional de semen i l'establiment de mesures per prevenir la transmissió. No obstant això, ni el risc de transmissió del virus pel semen, ni l'eficàcia d'aquestes mesures s'han avaluat quantitativament. L'objectiu de l'estudi va ser calcular mitjançant un model estocàstic, en cas d'introducció de llengua blava en un centre de recollida de semen boví (CRS), tant el risc de transmissió del VLlB a través de semen de boví, com la reducció d'aquest risc com a conseqüència de l'aplicació de les mesures preventives disponibles. El model es va aplicar a diferents escenaris, depenent per exemple del tipus de prova diagnòstica emprada, l'interval entre els controls dels toros donants, o la taxa de dispersió del VLLB dins del CRS. Els controls dels toros donants cada 60 dies per mitjà de la tècnica d'assaig per immunoabsorció lligat a enzims (ELISA) sembla ser un mètode poc eficaç per reduir el risc de transmissió del VLlB per semen en comparació amb l'ús de la reacció en cadena de la polimerasa (PCR) cada 28 dies. Un augment en la taxa de propagació del VLlB dins del CRS resulta en una reducció del risc de transmissió del virus per el semen. L'emmagatzematge de semen per 30 dies abans de la seva distribució sembla ser una forma eficaç de reduir el risc de transmissió per el semen. L'anàlisi de sensibilitat identificà la probabilitat d'excreció del VLlB en semen com un paràmetre crucial en la probabilitat de transmissió per semen. No obstant això, hi ha un alt grau d'incertesa associat a aquest paràmetre, amb diferències significatives en funció del serotip del virus.

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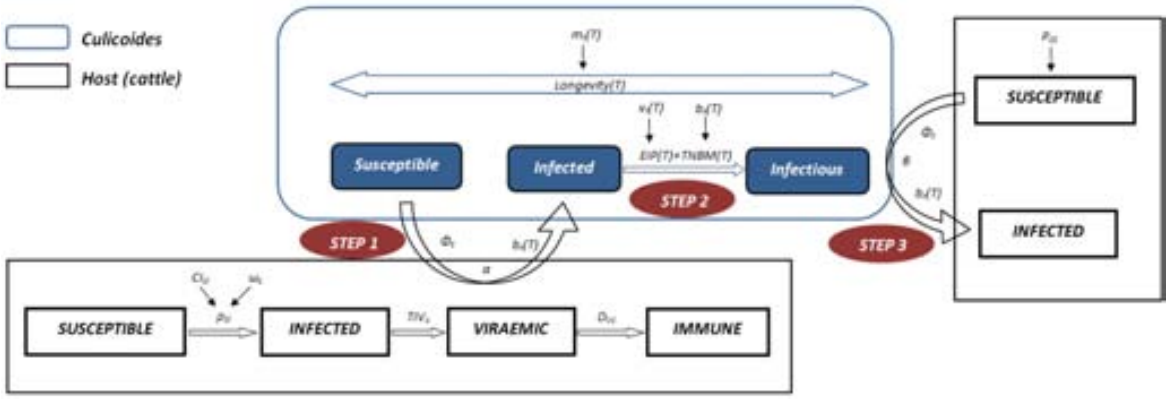
8. APPENDIX

APPENDIX

1. Risk assessment model

For overwintering to occur, a series of events (steps) have to take place (figure S1).



1.1. Probability of a Culicoides getting infected (STEP 1)

1.1.1 Probability Culicoides getting infected after one blood meal for month i (P_{ii})

1.1.1.1. Proportion of bites on cattle and on sheep

Φ_c: Proportion of bites on cattle

1.1.1.2. Probabilities of cattle (and sheep) being viraemic in month i

CI_{ci}: Cumulative incidence of cattle farms in month i

ω_c: Within farm incidence in cattle

ρ_{ci}: Probability of a cow being infected in month i

TIV_c: Time from infection to viraemia in cattle

D_{Vc}: Duration of viraemia in cattle

1.1.1.3. Proportion of bites on infectious host that infect a midge (α)

1.1.2. Probability Culicoides getting infected after n blood meals (P_{in})

$b_r(T)$: Biting rate

$m_r(T)$: Mortality rate

1.2. Probability a Culicoides survives the EIP and the TNBM: $P_{EIP+TNBM}$ (STEP 2)

$v_r(T)$: Virogenesis rate

EIP(T): Extrinsic Incubation Period

$b_r(T)$: Biting rate

TNBM(T): Time to the next blood meal

$m_r(T)$: Mortality rate

1.3. Probability of effective transmission (STEP 3)

1.3.1. The proportion of bites on cattle and on sheep

Φ_c: Proportion of bites on cattle

1.3.2. The proportion of cattle which were susceptible (P_{cs})

1.3.3. The proportion of bites per infectious midge that infect a host (β)

Figure S1: Diagram of the first 3 steps in the calculation of the probability of overwintering by pathways I & III

Those inputs (i.e. parameters that are fed to the model) which are general are presented in table SI. The outputs (i.e. parameters obtained by model calculations) which are general are presented in table SII. Input parameters specific of the German scenario are presented in table II (see study I).

Input parameter	Abbreviation	Value	Source	Section
Proportion of bites on cattle	Φc_{10}	*Pert (0.19; 0.60; 0.81)*	EOW	1.1.1.1.
Proportion of bites on sheep	Φs_{10}	*1-[Pert (0.19; 0.60; 0.81)]*	EOW	1.1.1.1.
Vector preference for cattle versus sheep	σ	*(1-Φc10)/ Φc10*	EOW	1.1.1.1.
Day of infection within a month	*DI*	*Uniform (1;30)*		1.1.1.2. & 1.5.
Time between infection and viraemia for cattle (days)	TIV_c	*Uniform (7;14)*	[2]	1.1.1.2.
Time between infection and viraemia for sheep (days)	TIV_s	*Uniform (1;6)*	[7]	1.1.1.2.
Duration of viraemia in cattle (days)	D_{Vc}	*Gamma (5; 4.12)*	[6]	1.1.1.2.
Duration of viraemia in sheep (days)	D_{Vs}	*Gamma (14; 1.17)*	[6]	1.1.1.2.
Mean probabilities of a cow being viraemic in month *i*,…, *i*+4 given infection in month *i*	*Various*	p_{vci}=0.20 p_{vci+1}=0.48 p_{vci+2}=0.02 p_{vci+3}=1.4 x10^{-4} p_{vci+4}=0.00	Simulation	1.1.1.2.
Days the cow remained viraemic in month *i*	d_{vci}	*Various*	Simulation	1.1.1.2.
Within farm incidence for cattle (and sheep)	ω_{ci} ($\&\omega_{si}$)	*Pert (0.25; 0.40; 0.6)*	[3]	1.1.1.2.
Mean probabilities of a sheep being viraemic in month *i*,…, *i*+3 given infection in month *i*	*Various*	p_{vsi}=0.38 p_{vsi+1}=0.23 p_{vsi+2}=5.8 x10^{-5} p_{vsi+3}=0.00	Simulation	1.1.1.2.
Days the sheep remained viraemic in month *i*	d_{vsi}	*Various*	Simulation	1.1.1.2.
Proportion of bites on infectious host that infect a midge	α	*Uniform (0.001; 0.15)*	[6]	1.1.1.3.
Mortality rate depending on the temperature (*T*)	$m_r(T)$	$0.009 \times exp(0.16 \times T)$	[5, 6]	1.1.2.1.
Biting rate depending on the temperature (*T*)	$b_r(T)$	$0.00017 \times T \times (T - 3.7) \times (41.9 - T)^{1/2.7}$	[8]	1.1.2.1.
Virogenesis rate depending on the temperature (*T*)	$v_r(T)$	$0.0003 \times T \times (T - 10.41)$	[8]	1.2.1.
Proportion of bites per infectious midge that infect a host	β	*Uniform (0.8; 1)*	[6]	1.3.3.
Time from start of *month -1* to the end of *PLVA* (in days)	TEP_{-1}	*30+ PLVA*		1.5.

Table SI: Model input parameters: abbreviations, values, sources from which the values were obtained and section in which they are referred to in the appendix. (EOW: Expert Opinion Workshop)

Output parameter	Abbreviation	Section
Probability of a *Culicoides* getting infected after one blood meal for month *i*	P_{Ii}	1.1.1.
Probability of a cattle being viraemic for month *i*	P_{Vci}	1.1.1.2.
Probability of a sheep being viraemic for month *i*	P_{Vsi}	1.1.1.2.
Probability of a *Culicoides* getting infected after *n* blood meals	P_{In}	1.1.2.
Daily probability of survival	P_S	1.1.2.1.
Day of the year *Culicoides* emerged (value obtained in a given iteration of the model)	*x*	1.1.2.1.
Probability of a *Culicoides* surviving day *x*	P_{Sx}	1.1.2.1.
Probability of a *Culicoides* surviving just one day (given emergence on day *x*)	P_{Sx1}	1.1.2.1.
Longevity of *Culicoides* (in a given iteration of the model)	*d*	1.1.2.1
Daily probability the vector has taken a blood meal on day *x*	P_{BMx}	1.1.2.2.
Probability of a *Culicoides* getting infected on day *x*	P_{Ix}	1.1.2.2.
Time to *Culicoides* infection	*TTCI*	1.1.2.3.
TTCI (value obtained in a given iteration of the model)	*r*	1.2.1.
Extrinsic Incubation Period	*EIP*	1.2.1.
Proportion of the *EIP* completed on day *x+r*	eip_{x+r}	1.2.1.
EIP (value obtained in a given iteration of the model)	*s*	1.2.1.
Time to the Next Blood Meal	*TNBM*	1.2.2.
Interval between blood meals	*IBBM*	1.2.2.
Proportion of the *TNBM* completed on day *x+r+s*	$tnbm_{x+r+s}$	1.2.2.
TNBM (value obtained in a given iteration of the model)	*t*	1.2.2.
Probability of surviving the *EIP* and the *TNBM*	$P_{EIP+TNBM}$	1.2.3.
Proportion of cattle which are susceptible	P_{cS}	1.3.2.
Probability of overwintering by persistence of BTV in cattle given infection of the cow the month before the start of the *PLVA* (month -1)	P_{c-1}	1.5

Table SII: Main model output parameters of the model: abbreviations, and section in which they are referred to in the appendix

1.1. Probability of a Culicoides getting infected

1.1.1 Firstly, the probability of a *Culicoides* getting infected after one blood meal for month *i* (P_{Ii}) was estimated as the product of: the proportions of bites on cattle and sheep (Φ_c and Φ_s, respectively); the monthly probabilities of cattle and sheep being viraemic (P_{Vci} and P_{Vsi}, respectively); and the proportion of bites on an infectious host that infect a midge (α).

1.1.1.1. Proportion of bites on cattle and sheep

The proportions of bites on cattle (Φ_c) and on sheep (Φ_s) were calculated as (Gubbins et al. 2008):

$$\phi_c = H_c / (H_c + \sigma H_s); \phi_s = 1 - \phi_c$$

Where H_c and H_s represented the cattle and sheep population in the affected area respectively (table II), and σ was a measure of the vector preference for cattle compared to sheep (if $\sigma < 1$, the vectors feed preferentially on cattle, and if $\sigma > 1$, the vectors feed preferentially on sheep). A hypothetical scenario of a farm with 10 cattle and 10 sheep, was used to obtain from the experts, an estimate of the proportion of vectors biting on cattle: Φ_{c10} (table SI), which was then used to calculate the value of σ.

1.1.1.2. Probabilities of cattle and sheep being infectious (viraemic) in month *i* (P_{Vci} and P_{Vsi} respectively), for *i*= November to April

First, the probability of a cow being viraemic in month *i* given infection in that same month (*i*): p_{vci} was calculated as:

$$p_{vci} = d_{vci} / 30$$

Where *30* represented the mean duration of a month in days, and d_{vci} represented the days the cow remained viraemic in month *i*, which was calculated as:

$$d_{vci} = 30 - (DI + TIV_c)$$

For: $0 \leq d_{vci} \leq D_{Vc}$

Where *DI* represented the day of infection within a month, which was modelled by a *Uniform (1; 30)* distribution; TIV_c the time between infection and viraemia for cattle (table SI); and D_{Vc} the duration of viraemia for cattle (table SI).

Similarly, the probability of a cow being viraemic in month *i+1* given infection in month *i* (p_{vci+1}) was calculated as:

$$p_{vci+1} = d_{vci+1} / 30$$

Where d_{vci+1} represented the days the cow remained viraemic in month *i+1*, which was calculated as:

$$d_{vci+1} = D_{Vc} - d_{vci}$$

For: $0 \leq d_{vci+1} \leq 30$

Likewise, the probabilities for months *i+2,…* were also calculated.

Monte Carlo simulations were used for the calculation of the probabilities (p_{vci}, p_{vci+1},..), and the results were used to construct empirical (non-parametric) cumulative distributions of the probabilities of a cow being viraemic in the different months after infection, which were used as inputs of the model. The mean values of these distributions are shown in table SI.

Then, the probabilities of a cow being viraemic for the months included within the study period (November to April: P_{VcNov} to P_{VcApr}) were calculated taking into account the probabilities of a cow being viraemic in month *i, i+1,..* given infection in month *i* (p_{vci}, p_{vci+1},..) and the probabilities of a cow being infected in months of August to April (as a cow infected in August may still be viraemic in November).

The probability of a cow being infected in month *i* (ρ_i) was calculated as:

$$\rho_{ci} = CI_{ci} \times \omega_c$$

Where CI_{ci} was the cumulative incidence of cattle farms in month *i* (table II), and ω_c the within farm incidence in cattle.

Similarly, using the specific inputs for sheep, the probabilities of a sheep being viraemic in the different months of the year (P_{Vsi}) were also calculated.

The probabilities of cattle and sheep being viraemic in November and December will determine the probabilities of infection of the vectors for pathways *Ia* and *IIIa*, while the probabilities of cattle and sheep being viraemic in January to April will determine the probabilities of infection of the vectors for pathways *Ib* and *IIIb* (figure 1).

1.1.1.3. Proportion of bites on infectious host that infect a midge: α (table SI)

Finally, the probability of a *Culicoides* getting infected after one blood meal for month *i* (P_{Ii}) was calculated as:

$$P_{Ii} = \left[\phi_c \times P_{Vci} \times \alpha\right] + \left[\phi_s \times P_{Vsi} \times \alpha\right]$$

1.1.2. Probability *Culicoides* getting infected after *n* blood meals (P_{In})

The longevity of *Culicoides* and the biting rate determine the number of blood meals the vector has taken and therefore its probability of infection.

1.1.2.1. Longevity of *Culicoides*

The mortality rate of *Culicoides* (m_r) depending on the temperature (T) was calculated as:

$$m_r(T) = 0.009 \times exp(0.16 \times T)$$

And the daily probability of survival (P_S) was calculated as:

$$P_S = exp(-m_r)$$

The vectors are not maintained at a constant temperature, and therefore mean daily temperature data was used to calculate the daily mortality rates and the daily probabilities of survival for the different days of the year.

The day of the year an adult *Culicoides* emerges will determine the values of all the temperature-dependent parameters which affect BTV transmission. Therefore, the probability of an adult midge emerging in each particular day of the year had to be estimated from the proportion of *Culicoides* trapped each month. In order to do that, the days from emergence to capture for month *i* (modelled by a *Uniform (1; d_i)* distribution, where d_i represented the mean longevity for month *i*), was subtracted from the day of capture within month *i* (modelled by a *Uniform (1; 30)* distribution), to estimate the proportions of the *Culicoides* trapped in month *i* that had emerged in month *i*, month *i-1*,….

Then, given a *Culicoides* which emerged in a particular day of the year (*x*), the probability that it survives just one day (i.e. until *x+1*): P_{Sx1}, was calculated as:

$$P_{Sx1} = (P_{Sx}) \times (1 - P_{Sx+1})$$

And the calculations of the probabilities associated to the survival of different number days are presented in table SIII. These values of days of survival and associated probabilities were used to construct a discrete distribution, which represents the longevity of the *Culicoides* emerged on day *x*. Based on (EFSA 2008), the maximum *Culicoides* longevity was set at 120 days. To account for the effect of low temperatures, when *Culicoides* were subjected to temperatures under 0ºC for 3 days within a period of 10 days, they were assumed to die (R. Meiswinkel personal communication based on experience in the field).

Longevity of *Culicoides* estimation	
Days of survival	Associated probability
0	$P_{Sx0} = (1 \quad P_{Sx})$
1	$P_{Sx1} = P_{Sx} \times (1 \quad P_{Sx+1})$
2	$P_{Sx2} = P_{Sx} \times P_{Sx+1} \times (1 \quad P_{Sx+2})$
120	$P_{Sx120} = P_{Sx} \times P_{Sx+1} \times .... \times P_{Sx+119} \times (1 \quad P_{Sx+120})$

Table SIII: Probabilities associated to the different days of survival used to construct the discrete distribution of *Culicoides* longevity

1.1.2.2. Biting rate

The *Culicoides* biting rate as a function of temperature: $b_r(T)$ may be calculated as:

$$b_r(T) = 0.000171 \times T \times (T - 3.6966) \times (41.8699 - T)^{1/2.7056}$$

The equation is only valid for temperatures above 4ºC (for temperatures below this value, transmission was assumed to stop).

Mean daily temperature data allowed the calculation of the biting rates for the different days of the year. Then, the daily probability the vector has taken a blood meal on day *x* (P_{BMx}) was calculated as:

$$P_{BMx} = 1 - exp(-b_{rx})$$

Where b_{rx} represented the biting rate for day *x*.

The probability of a *Culicoides* getting infected on day *x* (P_{Ix}) was calculated as:

$$P_{Ix} = P_{Ii} \times P_{BMx}$$

Where P_{Ii} was the probability of a *Culicoides* getting infected after 1 blood meal in month *i* (the month to which day *x* belongs).

Finally, for a *Culicoides* emerged on day *x*, and whose longevity is given by *d* days, the probability that the vector getting infected by BTV (P_{In}) was calculated as:

$$P_{In} = 1 - \prod_{j=0}^{d} (1 - P_{Ix+j})$$

1.1.2.3. Time to *Culicoides* infection (*TTCI*)

The *TTCI* in a given iteration (*r*) is obtained from:

$$r = Discrete(k; p_k)$$

Where *k* represents the days in which a *Culicoides* may get infected, and goes from 1 to *d* (longevity of the *Culicoides*) days; and p_k the probability of infection on day *k*. The values of the Discrete distribution for the calculation of the *r* of a *Culicoides* which emerged on day *x* are shown in table SIV.

Time to *Culicoides* infection (*r*) estimation	
Days of infection (*k*)	*Associated probabilities* (p_k)
1	$p_1 = (P_{Ix}) / (\sum_{j=0}^{d} P_{Ix+j})$
2	$p_2 = (P_{Ix+1}) / (\sum_{j=0}^{d} P_{Ix+j})$
d	$p_d = (P_{Ix+d}) / (\sum_{j=0}^{d} P_{Ix+j})$

Table SIV: Probabilities associated to the different days to infection used to construct the discrete distribution of the time to *Culicoides* infection (*TTCI*)

1.2. Probability a Culicoides survives the extrinsic incubation period (EIP) and the time to the next blood meal (TNBM)

In order to transmit the disease, the vector, once infected, needs to be able to survive the Extrinsic Incubation Period (*EIP*) and the Time to the Next Blood Meal (*TNBM*).

1.2.1. Extrinsic Incubation Period (*EIP*)

The virogenesis rate (v_r) depending on the temperature (*T*) may be estimated as:

$$v_r(T) = 0.0003 \times T \times (T - 10.4057)$$

The equation is only valid for temperatures above 11ºC, while for temperatures below this value, the virogenesis was assumed to stop.

The reciprocal of the virogenesis rate is the *EIP*, defined as the time between the infection of the vector and when it first becomes capable of transmitting the virus, was calculated as:

$$EIP(T) = 1 / v_r(T)$$

Mean daily temperature data was used to calculate the virogenesis rates (and the extrinsic incubation periods) which corresponded to the different days of the year.

A *Culicoides* which emerged on day *x* and got infected *r* days after emergence *(TTCI= r)*, was assumed to complete a proportion (eip_{x+r}) of the *EIP* on the day *x+r*:

$$eip_{x+r} = 1 / EIP(T_{x+r})$$

On the following day *(x+r+1)* the proportion of the *EIP* completed would be:

$$eip_{x+r+1} = 1 / EIP(T_{x+r+1})$$

The duration of the *EIP* for that *Culicoides* (*s*) would be given by the sum of the number of days needed so that the summatory of these proportions reaches one (i.e. the *EIP* is completed):

$$\sum_{j=x+r}^{\infty} eip_j = 1$$

1.2.2. Time to the Next Blood Meal (*TNBM*)

The reciprocal of the biting rate (b_r) is the interval between blood meals (*IBBM*), which for a given temperature *(T)* may be estimated as:

$$IBBM(T) = 1 / b_r(T)$$

Mean daily temperature data was used to calculate the biting rates (and the interval between blood meals) which corresponded to the different days of the year.

Once completed the *EIP*, in order to transmit the virus to a susceptible host, the vector has to take another blood meal. The time to the next blood meal *(TNBM)* for a given temperature *(T)* was modelled as:

TNBM (T) = Uniform (0; IBBM (T))

Therefore, the *Culicoides* which emerged on day *x*, got infected *r* days after emergence and needed *s* days to complete the *EIP* is assumed to complete a proportion ($tnbm_{x+r+s}$) of the *TNBM* on the day *x+r+s*:

$$tnbm_{x+r+s} = 1/TNBM(T_{x+r+s})$$

On the following day *(x+r+s+1)* the proportion of the *TNBM* completed would be:

$$tnbm_{x+r+s+1} = 1/TNBM(T_{x+r+s+1})$$

The duration of the *TNBM* for that *Culicoides* (*t*) would be given by the sum of the number of days needed so that the summatory of these proportions reaches one (i.e. the *TNBM* is completed):

$$\sum_{j=x+r+s}^{\infty} tnbm_j = 1$$

1.2.3. Probability of surviving the *EIP* and the *TNBM*

The probability of surviving the *EIP* and the *TNBM* ($P_{EIP+TNBM}$) was obtained by calculating the proportion of *Culicoides* for which the longevity *(d)* is bigger than the sum of the *TTCI (r)* plus the *EIP (s)* plus the *TNBM (t)*:

$$P_{EIP+TNBM} = P(d > [r + s + t])$$

1.3. Probability of effective transmission

Estimated independently for cattle and for sheep, taking into account:

1.3.1. The proportion of bites on cattle and on sheep

See section 1.1.1.1.

1.3.2. The proportion of cattle which were susceptible (P_{cS})

Calculated as one minus the proportion of the population immune. Immunity may have been achieved either after natural infection or by vaccination of the population against a specific serotype. As no vaccination was performed, the number of (naturally) immune cattle was estimated as the product of: number of cattle farms affected on the previous year, mean number of cattle per farm and mean within-farm prevalence (proportion of infected cattle within an infected farm). Then, the proportion of immune cattle was estimated by dividing the number of immune cattle by the cattle population. The proportion of sheep which were susceptible was also calculated. The values of the proportions of immune cattle and sheep for the German scenario are shown in table II.

1.3.3. The proportion of bites per infectious midge that infect a host (*б*)

In a given iteration, if *TTCI* (*r*) plus the *EIP* (*s*) plus the *TNBM* (*t*) was larger than the time from the emergence of the adult vector to the end of *PLVA*, overwintering occurred via the insect vector exclusively (pathway *I*) (figure 1). If not, it is necessary the contribution of the host to reach the next season (overwintering in the insect vector and the host, i.e. pathway *III*), and for that an extra step is needed: the viraemia of the host needs to go beyond the end of *PLVA*.

1.4. Probability the viraemia went beyond the end of the PLVA

When the time the virus spent on the host: time from infection to viraemia (*TITV*) plus duration of viraemia (D_{VC}), was larger than the time from BTV transmission to the host to the end of the *PLVA*, the viraemia of the host went beyond the end of the *PLVA* (figure 1).

1.5. Overwintering by persistence of the virus in the ruminant host

For overwintering to occur by persistence of the virus in cattle, the animal has to get infected before the *PLVA*, and then the viraemia has to last beyond the end of the *PLVA*.

Given infection of a cow the month before the *PLVA* (*month -1*), the probability of overwintering by persistence of BTV in cattle (P_{c-1}) was calculated as:

$$P_{c-1} = P\left(\left[DI + TIV_c + VP_c\right] > TEP_{m-1}\right)$$

Where *DI* was the day (within that month) the cow got infected, TIV_C the time from infection to viraemia in cattle, VP_C the duration of viraemia in cattle and TEP_{m-1} the time from start of *month -1* to the end of *PLVA* (figure S2).

The probability of overwintering by persistence of BTV in cattle for *month -2* (P_{c-2}) was also calculated. Similarly, the probabilities of overwintering by persistence of BTV in sheep for *month -1* and *month -2* (P_{s-1} and P_{s-2}, respectively) were calculated.

2. Sensitivity analysis

For linear regression models, the assumptions of independence, normality and constant variance of the residuals of the model were checked. Independence of the residuals was assessed by means of the Durbin-Watson estimate. Values in the range of zero indicated that the assumption was satisfied. The normality assumption was assessed graphically by obtaining a histogram of standardized residuals and a normal probability plot. Similarly, constant variance of residuals was assessed by obtaining a scatter plot of the regression standardized residual versus the regression standardized predicted value, and checking that there was not a clear pattern. Besides, correlation among independent variables was assessed by obtaining the measure of tolerance. Values above 0.6 were considered as acceptable. For logistic regression models, the correlation between variable was assessed using the Pearson's correlation coefficient. Values above 0.6 were considered as indicative of strong correlation.