

**Epidemiological analysis of the 2006 bluetongue virus serotype 8 epidemic  
in north-western Europe**

**Distribution and dynamics of vector species**

*R. Meiswinkel<sup>1</sup>, T. Baldet<sup>2</sup>, R. De Deken<sup>3</sup>, W. Takken<sup>4</sup>, J.-C. Delécolle<sup>5</sup>, Ph.  
Mellor<sup>6</sup>*

<sup>1</sup>CIDC-Lelystad, The Netherlands, <sup>2</sup>CIRAD, France, <sup>3</sup>ITM, Belgium, <sup>4</sup>WUR,  
The Netherlands, <sup>5</sup>Université Louis Pasteur, France; <sup>6</sup>Institute for Animal  
Health, U.K.

In the latter half of 2006 an unprecedented outbreak of bluetongue (BT) disease in sheep and cattle affected northern Europe spreading into five Member States (MSs) *i.e.* Belgium, France, Germany, Luxemburg and The Netherlands. For its dissemination bluetongue virus (BTV) is reliant on various species of biting midges of the genus *Culicoides*, which are the only known biological vectors.

To date four species of *Culicoides* have been incriminated as vectors in southern Europe; the most significant of these is the Afro-asiatic *C. imicola* responsible for at least 90% of BTV transmission in the Mediterranean Basin. It is regarded by some researchers to be a recent invader from Africa and to be spreading rapidly northwards threatening the livestock industry there. The three remaining vectors are endemic to the Palaearctic region but have till now played only a minor role in the spread of BTV; however, their importance appears to be increasing as BTV is moving northwards at a pace that is outstripping the slow advance of *C. imicola*.

The unforeseen arrival of BTV-8 in northern Europe soon raised a number of issues. The principal entomological questions posed were:

- Which vector *Culicoides* were involved, and
- Did these vectors include *C. imicola*?

In order to try and answer these questions vector surveys were quickly implemented in most of the affected countries. These commenced operation towards the end of August in France and were soon followed by similar studies in the remaining affected MSs. In all of these countries the collection of *Culicoides* continued well into December in parallel with outbreaks of the disease. The results of the various entomological investigations are summarised below:

- Not a single specimen of *C. imicola* was detected amongst a total of approximately 100,000 *Culicoides* collected in Belgium, France, Germany, Luxembourg, and The Netherlands.
- This demonstrates that species endemic to the Palaearctic region are quite capable of transmitting BTV and — judging from the rapid spread of the virus — it required no ‘pre-adaptive’ phase in indigenous *Culicoides*.
- In The Netherlands a novel, potential vector of BTV was discovered when a pool of 50 parous, non-engorged *C. dewulfi* were found RT-PCR+ to BTV-8. *C. dewulfi* breeds exclusively in the dung of cattle and horses; this irrevocable link with cattle and horses translates into added risk for livestock owners as *C. dewulfi* might be competent also in the transmission of some viral pathogens of horses. The OIE “important notifiable disease” African horse sickness which is caused by a virus related to BTV is probably the most important of these *Culicoides*-transmitted horse pathogens.
- The incrimination of *C. dewulfi* may explain the differing vectorial capacities reported amongst various populations of the Obsoletus Complex in the United Kingdom where the highest competency rates were linked to populations in which *C. dewulfi* occurred most abundantly. In turn this might explain — in part — why BTV did not spread in north-eastern France where the numbers of *C. dewulfi* were approximately 10x lower than those encountered in the Gulpen area of the south-eastern Netherlands and which was close to the Maastricht area of first infection (AFI).

- During a ‘snapshot’ survey of The Netherlands *C. dewulfi* was found to comprise >11% of the total *Culicoides* captured and occurred on 71% of the 108 farms surveyed nationally, which, after the Obsoletus Complex, made it the second-most prevalent taxon. Furthermore, light trap collections made nightly in the Gulpen area showed local populations of *C. dewulfi* to have a high parity rate i.e. 40% of the individuals captured comprised older females indicating that their survival rate was high and, also, that they were feeding repeatedly (these two elements in the life cycle of *Culicoides* being crucial to the successful replication and subsequent transmission of BTV). In Belgium there is some evidence to suggest that *C. dewulfi* has expanded its range in the last 50 years; however, this may be an artefact of the different sampling methods used over time or it may be due to the low emphasis placed on the collection of *Culicoides* in the vicinity of livestock in the past.
- The belated discovery of *C. dewulfi* as the fifth species now suspected to be involved in the transmission of BTV in Europe owes much to the fact that very few researchers are able to identify it with confidence and instead lump it under the Obsoletus species complex. This illustrates the need to continuously develop the level of taxonomic expertise that currently exists within Western Europe and where the number of active *Culicoides* taxonomists can be counted on one hand.
- Subsequent to the virological findings made in regard to *C. dewulfi* three pools of parous mixed *C. obsoletus/C. scoticus* collected on the same farm in Crapoel in the south-eastern Netherlands, were found also to be RT-PCR+ to BTV-8. This is the clearest evidence we have that more than one species of *Culicoides* was involved in the outbreak of BT across northern Europe. Thus the particular climatic conditions that prevailed in the area at the time of the outbreak, which made it significantly warmer than at any time in recorded history, appear to have been such as to promote the successful replication of BTV in and transmission by more than one species of vector. A lesson to be learned here is that in the future the continuation of climate warming is likely to enhance the vector potential of still other species of *Culicoides* endemic to the region.
- For this reason it is essential that the veterinary authorities in all affected and adjoining MSs initiate comprehensive vector surveillance programmes to identify which species of *Culicoides* occur abundantly in the vicinity of all major breeds of livestock and to determine their seasonal profiles. These surveys will help identify regions in which multiple vector species occur (as was found for 10% of the farms surveyed in The Netherlands) and may highlight areas that are vector-free and thus suitable for the “quarantining” of livestock to facilitate the development of a safe disease-free export trade in ruminants.
- In this context it is of concern to note that all except one (*C. imicola*) of the previously identified vectors of BTV in southern Europe are now known to occur also widely across northern Europe. In The Netherlands, for example, a ‘snapshot’ survey revealed species of the Obsoletus Complex (including *C. obsoletus* and *C. scoticus*) to be exceptionally widespread occurring in 94% of the light trap collections made on 108 cattle farms sampled nationally. Additional studies made in the affected MSs in northern Europe have confirmed the widespread dominance of the Obsoletus Complex on livestock farms; in

addition — and like *C. dewulfi* — this insect complex had a high parity rate of 40%, which increases the likelihood of it having played a significant role in the outbreak of BTV a supposition that is supported by the three RT-PCR+ results mentioned above.

- The most recent entomological data to emerge is that low numbers of adult *Culicoides* principally of the Obsoletus Complex, and including freshly bloodfed individuals, have on occasion been captured in light traps operated throughout the winter (January, February and March 2007) in various MSs in northern Europe. In all likelihood this persistent activity of adult *Culicoides* owes much to the mild temperatures that have continued to prevail across northern Europe. However, the numbers of midges that have remained active appear to have been too few to sustain the BTV transmission cycle at the time of writing in March 2007. However, it would be premature to conclude that BTV did not overwinter in the region and therefore vigilance must remain high.
- In regard to midge abundance levels it is notable that >1 000 *Culicoides* were found in fewer than 5% of the approximately 500 light trap collections made to date throughout northern Europe and when using mostly the ‘golden standard’ Onderstepoort-type blacklight trap. In The Netherlands, during the ‘snapshot’ survey conducted in September 2006, an average of 333 *Culicoides* was captured/trap night. Of these, only half belonged to vector species, which, on average, is 40-180x lower than the average vector densities encountered during the same month in parts of the Mediterranean Basin where BTV was also circulating. This finding can be interpreted as follows:
  - It may indicate that comparatively low mean *Culicoides* densities were able to maintain a very efficient level of BTV circulation across northern Europe, and/or
  - It may indicate that vector densities as estimated from light trap collections are a gross underestimate of the actual numbers of *Culicoides* attacking livestock, possibly because midges may bite also during the daytime particularly on days when it is overcast and when light intensity levels are low but at which times light traps are not usually operated.

The proportion of the midge populations involved in daytime attacks is not known but if it is large this could have important implications for the diurnal transmission of BTV. For obvious reasons it is not possible to monitor diurnal *Culicoides* activity using light traps; different capture methodologies will have to be implemented to obtain this kind of information in the future (e.g. bait animals, suction traps, CO<sub>2</sub> traps).

- Various attempts (both in France and in The Netherlands) failed to incriminate species of the Pulicaris Complex in the transmission of BTV. In northern Europe the Pulicaris Complex comprises at least six species and when these are counted together this can result in the complex being recorded as the dominant taxon in some areas. However, previous work, elsewhere, has incriminated only one of the constituent species of the Pulicaris Complex i.e. *C. pulicaris sensu stricto* (*ss*) as being involved in the transmission of BTV and in northern Europe seems less abundant than some of the other members of the complex such as *C. punctatus* and *C. newsteadi*. Indeed, according to the data collected thus far *C. pulicaris ss* comprised a mere 3-6% of all the *Culicoides* captured and its distribution was limited so that it occurred on only 14% of the 108 farms surveyed in The Netherlands. Taken as a whole therefore, the vector surveillance studies indicate *C.*

*pulicaris ss* played no significant role in the northern European BT outbreaks possibly because of its low abundance and restricted distribution in the area.

- It is worth examining also, and in greater detail, additional data collected on *C. pulicaris ss* in Crapoel (The Netherlands) and in north-eastern France. In Crapoel it comprised 15% of the *Culicoides* captured outside cattle sheds and in France 11%. However, inside cattle sheds it comprised, respectively, only 0.2% and 0.3% of the thousands of *Culicoides* captured. These data may mean that:
  - *C. pulicaris ss* is strongly exophilic and preferentially feeds only on cattle that are maintained outdoors;
  - *C. pulicaris ss* is exophilic and rarely feeds on cattle preferring some unknown alternative host such as birds; or
  - *C. pulicaris ss* feeds regularly on cattle but does so mostly diurnally and for this reason it is not being captured in significant numbers in light traps which operate efficiently only in the dark.

These various possible interpretations demonstrate how little knowledge exists on the daily life cycle and biting habits of the vector *Culicoides* of northern Europe and they require detailed ecological investigations to resolve.

- As noted above at least six species of the Pulicaris Complex occur in The Netherlands and it is likely that a similar situation applies also in the other MSs in the region. During the recent ‘snapshot’ survey in The Netherlands the geographic ranges of all these species were mapped and showed most to have distinct patterns of occurrence, which are a reflection of their specific biologies. For example, *C. halophilus* was found in association with saline coastal habitats whereas *C. impunctatus* — represented by a single specimen — was found only in the vicinity of peat bogs. This preference for bog-lands was confirmed in a separate study conducted in The Netherlands in which it was found that *C. impunctatus* occurred also in wetlands and that it was on the wing only in the first half of summer. This finding suggests that *C. impunctatus* played no significant role in the epidemiology of the 2006 BT outbreak which occurred in the latter part of the year at a time when this species was no longer active. These observations highlight the fact that each taxon within the Pulicaris Complex inhabits an almost exclusive biotope, which will influence greatly its distribution both in space and time and hence its potential involvement in any BTV outbreak. This makes it imperative to be able to identify each of the taxa accurately in order to be able to interpret field data correctly and provide appropriate advice to the veterinary authorities.
- In an effort to reduce the impact of outbreaks of BTV the competent authorities recommended that livestock be housed at night in the belief that this would reduce significantly the *Culicoides* attack rate (and thereby lower the BTV transmission rate). All farmers within the 20 km infection zone were therefore compelled to keep their animals indoors each night and to treat the animals monthly with a pour-on insecticide. But in France and in The Netherlands it has been discovered that *Culicoides* enter animal housing quite freely. Of particular concern, is that >95% of these comprised the vector species *C. obsoletus* and *C. dewulfi*. Work in these two MSs showed that early in the season when night-time temperatures remained high larger numbers of *Culicoides* were captured in light traps operated outside stables. However, later in the season, when the temperatures began to drop to single digits, a reversal occurred and more *Culicoides* were

captured both earlier in the evening and inside rather than outside stables. These data suggest that during the cooler times of the year *Culicoides* emerge from their resting places sooner in the day (when it is still reasonably warm) probably to attack livestock while still at pasture. It is well known that species such as *C. obsoletus* will intensify their attacks on overcast days when low-light conditions prevail. In such situations it is possible that attacking *Culicoides* may follow the cattle returning to their milking sheds and accompany them indoors. Once inside, the biting midges would then be able to complete their blood feeding activities, only to be captured subsequently in the light traps operated nearby. This sequence of events would explain why increased numbers of midges were captured inside animal houses late in the season and why a high percentage of them were freshly bloodfed.

- In order to protect housed animals from attack by *Culicoides* it may require that such housing be sealed to a level where the lack of circulation of air might become a welfare problem or which is economically not viable. But even if such well-sealed buildings were to be ventilated perhaps by screening with insect-proof mesh this would do little to prevent *Culicoides* from entering the housing along with cattle in the late afternoon (as described above). To control this possible influx by *Culicoides* would require the installation of walk-through insecticidal sprayers. Overall, there is a paucity of information on the behavioural activities of vector species of *Culicoides*, especially in relation to their interactions with host animals and their biting activities. Detailed data are urgently required before clear and reliable recommendations can be provided to the veterinary authorities on the subject.
- The discovery of significant numbers of *Culicoides* in buildings towards the end of the season has raised two additional points of concern:
  - Do some species of *Culicoides* breed indoors? and
  - Can BTV-infected late-season adult *Culicoides* overwinter inside cattle sheds to emerge months later in spring to initiate a recrudescence of the BTV transmission cycle?

These questions have yet to find answers.

- The discovery that larger numbers of *Culicoides* may be found indoors than outdoors, and especially towards the end of the season, demands that the earlier attempt to declare the vector-free period to be when "...<10 *Culicoides* are found in a light trap suspended outdoors for one night" be refined. This criterion could be amended to include the age-grading of the captured *Culicoides* so that a light trap must contain "... <5 parous *Culicoides*" as it is the older individuals that represent a threat both in terms of their longevity and their potential to harbour the virus of BT and to transmit it. However, research must be continued to obtain more robust data on vector competence rates, transmission rates and vector ecology in the northern European context, in order to be able to define the vector-free season with greater certainty.

It is now evident that the *Culicoides* fauna endemic to northern Europe harbours multiple vectors of BTV. As vectors of BTV in other parts of the world have been shown to transmit a range of other viral pathogens of livestock (African horse sickness virus, Akabane virus, epizootic haemorrhagic disease virus, equine encephalitis virus) this suggests that such pathogens may be

transmitted if they were to be introduced into northern Europe during climatically favourable periods.

## **Annex A to Appendix 9**

### **Longitudinal sampling of *Culicoides* spp. in order to determine the risk of bluetongue outbreaks in The Netherlands**

***W. Takken<sup>1</sup>, N. Verhulst<sup>1</sup>, E-J. Scholte<sup>1</sup>, F. Jacobs<sup>1</sup>, Y. Jongema<sup>1</sup>, A. de Koeijer<sup>2</sup>,  
H. van Roermund<sup>2</sup>, R. van Lammeren<sup>1</sup>.***

**<sup>1</sup>Wageningen University, Wageningen, The Netherlands; <sup>2</sup>Wageningen University and Research Centre, Lelystad, The Netherlands**

## Summary

Emerging infectious diseases are a recognized threat to the health of humans and animals. Causes of this development are manifold, but rapid environmental change and climate change as well as increased international trade and travel are considered major determinants of the arrival of such diseases. The Netherlands has enjoyed a relatively disease-free state for more than one century, aided by the development of effective drugs and vaccines and installation of stringent hygienic measures. In order to be prepared for the potential outbreak of vector-borne diseases of livestock and humans, a study was undertaken to investigate the presence and distribution of candidate vectors of infectious diseases, with emphasis on bluetongue vectors (*Culicoides* spp.) and develop a risk model for the spread of the disease once it had arrived. The study focused primarily on the relationship between characteristic ecosystems suitable for bluetongue vectors and climate, as well as on the phenology and population dynamics of these vectors.

Twelve locations were selected, distributed over 4 habitats: a wetland area, 3 riverine systems, 4 peat-dominated nature reserves and 4 livestock farms. *Culicoides* populations were sampled continuously using CO<sub>2</sub>-baited counterflow traps from July 2005 until August 2006, with an interruption from November 2005 – March 2006. All vectors were identified to species. Meteorological and environmental data were collected at each location.

*Culicoides* species were found in all four different habitat types studied. Wetland areas and peat bogs were rich in *Culicoides* spp.. The taxonomic groups *C. obsoletus* and *C. pulicaris* were significantly associated with such farms. Eighty eight percent of all *Culicoides* consisted of the taxon *C. obsoletus/C. scoticus*. On the livestock farms, 3% of *Culicoides* existed of the suspected bluetongue vector *C. dewulfi*. *C. impunctatus* was strongly associated with wetland and peat bog. The BTV risk model demonstrates that under favourable climate conditions, such as in 2006, bluetongue virus can spread in The Netherlands, aided by the presence of suitable vectors. These vectors are indigenous and not exotic.

The study demonstrates that candidate vectors of bluetongue are naturally present in a wide range of habitats in The Netherlands, distributed widely across the country. Under favourable climatic conditions, following virus introduction, bluetongue can spread among livestock (cattle, sheep and goats), depending on the nature of the viral serotype. The question now arises whether the virus can survive the winter conditions in north-western Europe and whether measures can be taken that effectively halt further spread of the disease.

## Introduction

In recent years, the livestock industry in Europe has suffered from a number of outbreaks of zoonotic diseases that were thought to have been eradicated or, at least, kept well under control. Bovine spongine encephalitis (BSE), swine fever, foot and mouth disease and chicken flu have caused havoc among the livestock industry and huge losses to the European Community. These diseases have also affected the human society, in several cases causing the death of veterinary staff and disrupting traffic and trade by stringent control of transport into and out of disease-affected areas. Many farmers were forced to have their livestock cleared for the duration of the epidemic, and as a result suffered economic losses and traumas because of the death and disease surrounding their environment.

In addition to these disease outbreaks of “old” animal diseases, new and hitherto unknown diseases have also emerged, such as SARS and the Asian chicken flu as well as granulocytic Ehrlichiosis and rickettsiosis. In addition, Lyme disease (caused by the *Borrelia* parasite), leishmaniasis and tick-borne encephalitis are rapidly increasing in incidence throughout Western Europe. Bluetongue, a viral disease affecting sheep and cattle, has become established in southern Europe, in particular after its accidental introduction in Italy in 1998 (2005). In the United States the arrival of West Nile virus has caused huge losses to the horse-racing industry, while this disease has also claimed many human lives. In Europe, West Nile is occasionally being reported, particularly in Italy and France.

Although several of these diseases have been known for many years, and several are not vector-borne, it is clear that these diseases are reported much more frequently in the last decade compared to the preceding 50 years. Some of these diseases have never been known to occur in Europe. The European Community has developed a common system for reporting harmful infectious diseases, and protocols for the intervention of disease outbreaks are kept at the ready by all member states. Sadly, this does not prevent that some of them return periodically, with grave consequences. Whereas the air-borne and communicable diseases can sometimes be controlled by vaccination and quarantine, this is less clear for vector-borne diseases.

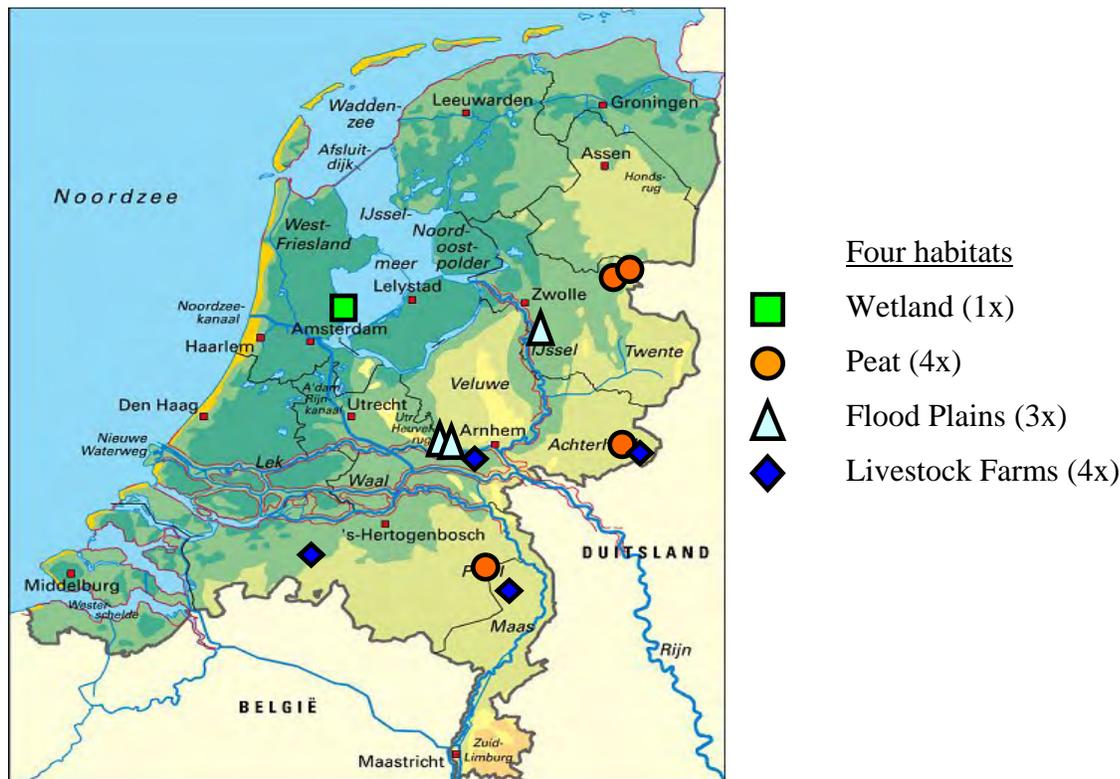
The increasing occurrence and prevalence of the vector-borne zoonoses are being attributed to environmental change (mostly caused by climate) and increased movement of livestock from one country to the other. In addition, vectors can rapidly spread across regions with favourable winds, with trucks and as ectoparasites on pets (dogs, cats, birds). The open border policy of the European Community makes control of animal movements virtually impossible. Climate change is one of the other issues that might contribute to the rapid spread and increased incidence of vector-borne diseases. Vectors profit from higher summer and winter temperatures by extending their reproductive season and greater ability to disperse. Furthermore, the expansion of nature reserves in many countries coupled with restricted hunting laws have caused an explosion of wildlife such as roe deer, red deer and wild hog. Although rabies (a communicable disease, not vector-borne) has been eradicated from Western Europe by a combined effort, many other wildlife diseases still abound and could become more prevalent as a result of the good conditions for wildlife.

Enzootic diseases such as West Nile and Ockelbo are rare or non-existent in The Netherlands, but they are widely present elsewhere in Europe. By contrast, Lyme disease, Ehrlichiosis, Babesiosis and tick-borne rickettsiosis are widely present, and still increasing in incidence. The latter is presumably due to favourable conditions for the tick vector of these parasites, *Ixodes ricinus*. It has been noted that increasingly domestic livestock and dogs are affected by Lyme disease. Because of the rising ambient temperatures and increased rainfall, environmental conditions for the vectors of these diseases are becoming better, and this may result in higher vector densities and/or longer transmission seasons.

Following the widespread reports of BTV in Southern Europe in recent years, the Dutch government commissioned a study to investigate the presence and distribution of *Culicoides* spp. in The Netherlands with emphasis on likely areas where such vectors would be readily found. Vector populations of different habitat types have been compared, and associated with environmental variables such as climate and vegetation. The data were used to develop a BTV risk model.

## Study Areas

Since almost all arthropods of human and veterinary vector-borne diseases are closely associated with humid or wet areas, these types of areas are considered most at risk for vector-borne disease transmission, especially when there are large mammals present (**Figure 1**). These areas include especially fresh water-associated semi natural areas: wetlands, peat areas and floodplains, with free-living large domestic animals such as horses and cows. These different areas are regarded as three different arthropod vector ‘habitats’. A fourth, less natural, habitat is a livestock farm with domestic animals. This habitat was included because of the existing day-to-day interactions between arthropods, domestic animals, and people.



**Figure 1 - Study-areas**

Twelve study sites were selected in The Netherlands: one wetland area, four peat areas, three floodplain areas, and four livestock farms, all of which are described in detail below. Of these study sites geographical and ecological details were determined. The ecological characterization of the sites included description of the type of vegetation at various layers (trees, shrubs and herbs), determining all occurring plant species, and estimate their cover percentages. Additionally, the landscape of each site was described. These data, combined with the entomological data of collected haematophagous arthropods at these habitats, provide the basis for a risk assessment for transmission of vector-borne infectious diseases in The Netherlands.

### *Wetland (Oostvaardersplassen)*

The Oostvaardersplassen is an important area for (migratory) birds and there are large herds of Heck cattle, Konik horses and red deer. Migratory birds can bring diseases from Southern Europe and Africa to The Netherlands. Many blood sucking insects are opportunistic feeders (feeding on birds and mammals) and can therefore transmit diseases from migratory birds to the cattle, horses and deer.

The area consists of large wet areas with common Reed (*Phragmites australis*), which is alternated with small drier areas with willows (*Salix alba*) and nettles (*Urtica dioica*).

### *Peat bogs & moores*

Like the wetland area, the peat bogs were also very wet and large numbers of mosquitoes (Diptera; Culicidae), midges (Diptera: Ceratopogonidae) and ticks (Acari: Ixodidae) were found. In some peat areas, these large numbers of blood sucking insects can be of great nuisance.

Three study areas were selected, but four sites were sampled. Because the Engbertsdijksevenen consist of a wet and a more dry area, two sites were selected in this area, one in the north of the area, and one in the south-western corner.

- Engbertsdijksevenen South West (SW) (Overijssel)

Mosquitoes have been of great nuisance in this area. Groundwater levels have been modified to reduce the mosquito population and other studies were carried out to investigate the problem. (Verdonschot 1996).

The Engbertsdijksevenen (SW) site is wetter than the Engbertsdijksevenen North East (NE). The water level is maintained by dykes. Ticks could only be sampled just outside the dykes, because on the 'wet' side, the area was inaccessible for men and not suitable for tick monitoring.

The vegetation mainly consisted of peat moss (*Sphagnum*) and in the drier areas of birch (*Betula*) and *Myrica gale*, which is often associated with midges (*Culicoides spp.*) (Blackwell et al. 1999)

- Engbertsdijksevenen (NE) (Overijssel)

The Engbertsdijksevenen (NE) is drier than the site in the south. There is a herd of Limousin cattle that keeps the grass short. *Myrica gale* is not present, there is less *Sphagnum* and more *Betula* trees.

- Korenburgerveen (Oost-Gelderland).

The area consists of flooded forest with alder (*Alnus glutinosa*), ash (*Fraxinus excelsior*) and oak (*Quercus robur*) altered with meadows with Galloway cattle. Other vegetation present: common reed (*Phragmites australis*), blackberry (*Rubus fruticosus*), rowan (*Sorbus aucuparia*) and nettles (*Urtica dioica*.)

- De Grote Peel (Noord-Brabant/Limburg).  
An open area with small pieces of birch (*Betula*) forest. Animals include deer, sheep and Limousin cattle. Most of the ground vegetation consisted of purple moor grass (*Molinia caerulea*)

#### *Floodplains*

These consist of river flood plains that are flooded several times a year during times of excessive rainfall in Germany/France. These plains contain many shallow water bodies, which are potential breeding places for mosquitoes (*Culicidae*)

- Duursche Waarden (Overijssel).  
An area with Pony's and Highland cattle. Parts of the area are flooded several times a year. Vegetation consists of several willow (*Salix*) species, purple loosestrife (*Lythrum salicaria*) and bentgrass (*Agrostis stolonifera*)
- Afferdense Waarden (Gelderland).  
A relatively new nature reserve with horses and large open areas. Vegetation consists of a few *Crataegus* shrubs, bentgrass (*Agrostis stolonifera*) and water mint (*Mentha aquatica*).
- Blauwe Kamer (Utrecht).  
A small nature reserve with Galloway cattle and Konik horses. Vegetation consists preliminary of *Crataegus* and nettles (*Urtica dioica*).

#### *Ecological livestock farms*

In order to collect a large diversity of arthropods, it was decided to focus the study of insects and ticks in relation to farming areas on ecological farms. On ecological farms it is not allowed to use pesticides and these farms are therefore ideal for monitoring species and populations of arthropods. On non-ecological farms pesticide use will influence the results and analysis of data more difficult because of frequent and irregular interventions. The data on ecological farms, presented in this report, do not say anything about the difference in the risk of arthropod diseases between ecological and non-ecological farms. All farms were mixed farms, with the production of cash crops and livestock.

- Ecological farm 'De Brömmels' (Winterswijk, Oost-Nederland).  
A small goat farm where, apart from goats, also pony's (3), cows ( $\pm 10$ ), sheep (2) and pigs (2) were present. The farm also includes a camping, where many people are present during summer holidays. Swallow nests (migratory birds), a small lake and other small water bodies, which can be a potential breeding site for mosquitoes, are present. The surrounding pastures are alternated with small forests. Flying bloodsucking insects were collected in the pastures, and ticks were sampled in a small forest next to the farm.
- Ecological farm 'De Voortse Hoeve' (Hilvarenbeek, Zuidwestelijk Noord-Brabant). Op 'De Voortse Hoeve' is a pig farm and no other farm animals were present. The pigs are both indoors and outdoors. The insect traps were standing outside, but under a small roof.
- Ecological farm 'De Waog' (Neer, Midden-Limburg).  
Vegetables are grown on this farm, but also a few sheep ( $\pm 10$ ) were present. 50 meters from the farm dogs, horses, cows and sheep were present.

- Ecological farms 'De Ravenswaard' (Afferden, Zuid-Nederland).

This dairy farm lies one kilometer from the River Waal. Cows walk outside, or are inside an open barn. There is one horse. Swallow nests (migratory birds) are present and a few potential breeding sites are present.

## Materials and Methods

### *Monitoring methods*

Carbon-dioxide baited counterflow traps (Liberty Plus®, American Biophysics Co., Greensborough RI, USA) were used for all sampling studies of *Culicoides*. These traps have proven reliable sampling tools for mosquitoes and biting midges in the USA and Scotland (Kline 2002, Mands et al, 2004). The traps produce a constant flow of carbon dioxide, which mimics the breath of mammals. The traps ran continuously and were being emptied once a week

### *Diagnostics of insect samples*

Weekly collections were killed at -20°C, and transferred to 70% ethanol. Samples were identified to the nearest taxon using keys kindly provided by S. Carpenter, Institute of Animal Health, Pirbright, UK and with information provided by Meiswinkel et al. (2004) and Gomulski et al. (2005).

### *Recording of meteorological data*

Tinytag® meteorological data loggers (Gemini Data Loggers, Chichester, UK) were used to record humidity (min, max, mean) and temperature (min, max, mean) every ten minutes. Because of problems with the dataloggers in 2005, data was only available for 2006. To be able to compare data of 2005 and 2006 interpolated data from established meteorological field stations were used.

Temperature data was available from 46 weather stations of MeteoConsult (ALWeer database, Alterra, Wageningen UR). Because insects were monitored once a week, data was converted to average week temperatures and maximum and minimum per week.

The data of the weather stations were interpolated (Spline) to a climate map of The Netherlands for 2005-2006. This map was used to determine the temperature at the 12 monitoring locations.

### *Classification of vegetation structures*

An index of the vegetation was made according to the method of Blanquet (adjusted by Maarel). This index was used to classify the vegetation types ((E Weeda 1981, EJ Weeda et al. 2005) of the 12 sampling sites (appendix 7).

## Results

During the study, fifteen species of *Culicoides* (biting midges)(Diptera: Ceratopogonidae) were collected. The key species were:

- *Culicoides impunctatus*: A very common *Culicoides* species in The Netherlands (this report) and proven to be orally susceptible to bluetongue in a laboratory study (Carpenter et al. 2006).
- Subgenus *Avaritia*: this subgenus comprises the taxa *C. obsoletus*/*C. scoticus*, *C. dewulfi* and *C. chiopterus* as well as the African vector *C. imicola* (Gomulski et al. 2005). *C. obsoletus*

and *C. scoticus* have been incriminated as bluetongue vectors in Italy (De Liberato et al. 2005); *C. dewulfi* is the only species from which bluetongue virus was identified during the bluetongue outbreak in The Netherlands of 2006<sup>1</sup> although in a *C. obsoletus* pool in Germany viral DNA was also detected.

- Subgenus *Culicoides pulicaris*. This is a species complex, including the species *C. pulicaris* and *C. punctatus*: A known bluetongue virus vector (Purse et al. 2004), which also occurs in colder areas like The Netherlands.

A complete list of all species collected during this study is presented in **Table 1**.

**Table 1** *Culicoides* spp. collected during the longitudinal study of the 12 locations, 2005-2006.

<b>Species</b>
<i>Culicoides impunctatus</i>
<i>Culicoides minutissimus</i>
<i>Culicoides nubeculosis</i>
<i>Culicoides obsoletus/scoticus</i>
<i>Culicoides chiopterus</i>
<i>Culicoides dewulfi</i>
<i>Culicoides festivipennis</i>
<i>Culicoides odibilis</i>
<i>Culicoides pulicaris</i>
<i>Culicoides punctatus</i>
<i>Culicoides newstaedy</i>
<i>Culicoides fascipennis</i>
<i>Culicoides maritimus</i>
<i>Culicoides achrayii</i>
<i>Culicoides stigma</i>

#### *Wetland*

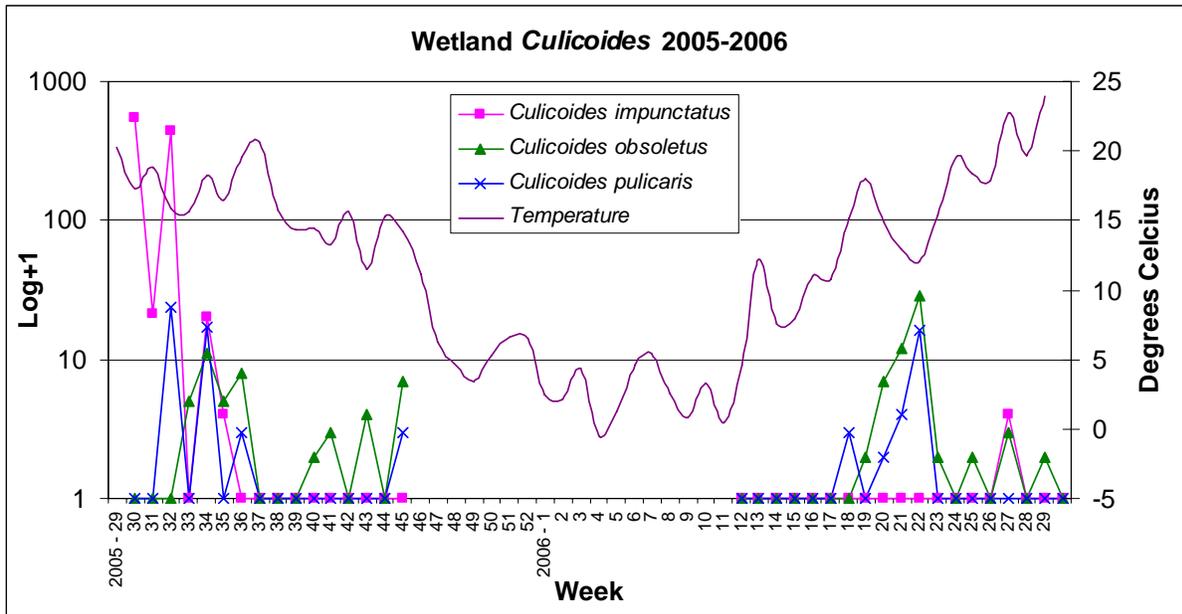
Of the total of 15 *Culicoides* species found during this study, ten species were found in the wetland area. Numbers of *Culicoides* were high in 2005 compared to the other habitats, but were much lower in 2006. This difference in *Culicoides* numbers between 2005 and 2006 at this site is due to the very high number of *Culicoides impunctatus* caught in week 29 of 2005 (**Figure 2**). By contrast, numbers of *C. impunctatus* were very low in that same week in 2006. These data show that numbers of *C. impunctatus* can differ much within seasons and between years.

It is assumed that most *Culicoides* species hibernate as larvae in The Netherlands. Our data seem to support this assumption. Unfortunately it is not possible to tell how long this hibernation period was because sampling was not prolonged during the winter months. WE assumed with our sampling on 1 March 2006, and adult *Culicoides* spp. were not found until week 18 (first week of May), which could be temperature-dependent, corresponding with the temperature increase

---

<sup>1</sup> In one pool of *C. dewulfi*, collected by Dr. van Meiswinkel and Dr. Goffredo in Limburg Province in September 2006, genetic material of BTV serotype 8 was indentified. Source: Dr. P. van Rijn, WUR-ASG, personal communication

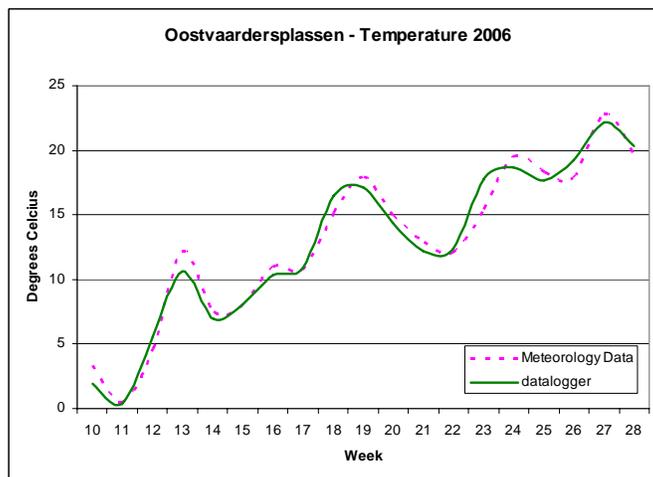
(above 10 °C) in week 18. The spring of 2006 was relatively cold, with many occasions of night frost.



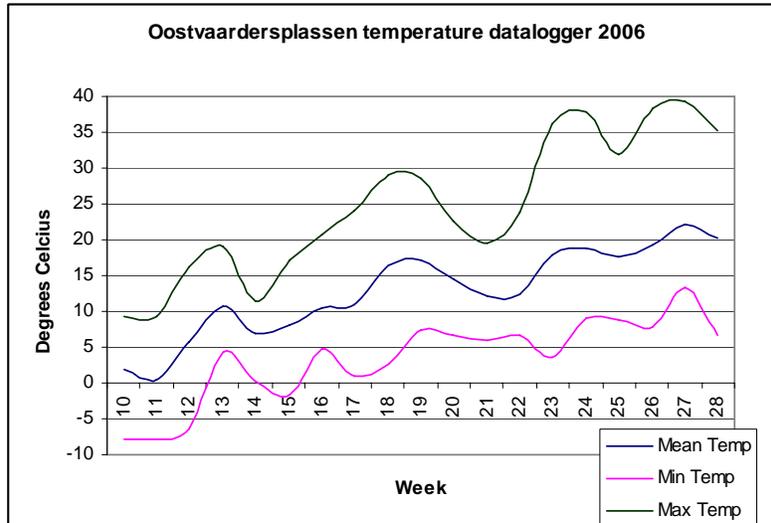
**Figure 2 - Wetland: catches of three most abundant *Culicoides* species**

*Meteorological data wetland*

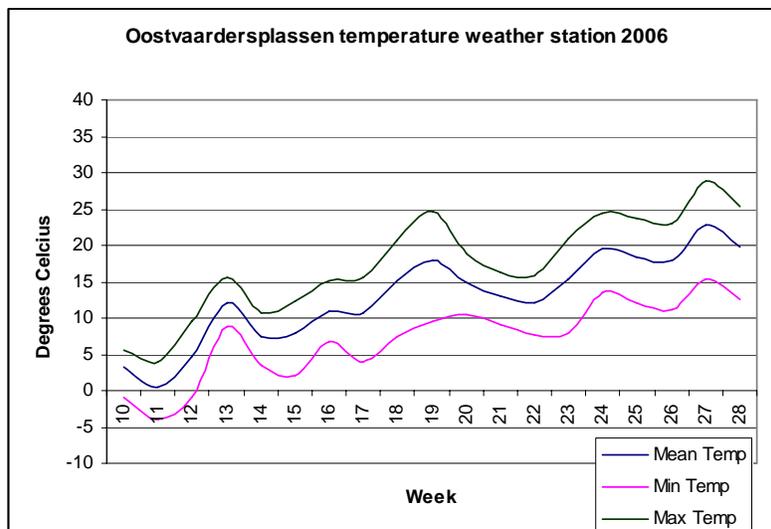
Since data logger results were not available in 2005, mean temperatures per week presented in **Figure 3** were interpolated from weather station data. In **Figure 3** these interpolated data are compared with the data from the data logger collected in 2006. Although **Figure 3** shows that these temperatures were very similar, **Figure 4** and **Figure 5** show that there was a distinctive difference when maximum and minimum temperatures are compared. Although mean temperatures were similar at microclimate levels compared to those from the weather stations, temperature fluctuations were much higher at the level of microclimate. This can be of major importance for insect development and survival.



**Figure 3 Wetland area (Oostvaardersplassen): mean temperature data from datalogger compared with interpolated meteorological data**



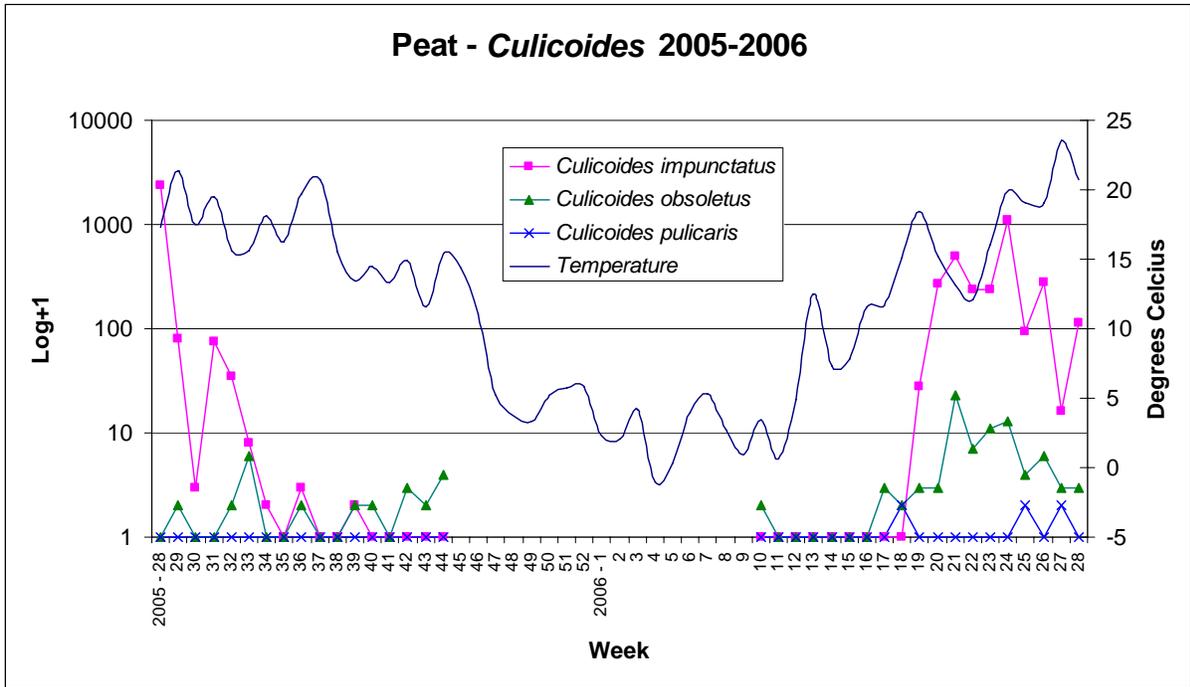
**Figure 4 Wetland area (Oostvaardersplassen): mean, maximum and minimum temperatures from datalogger.**



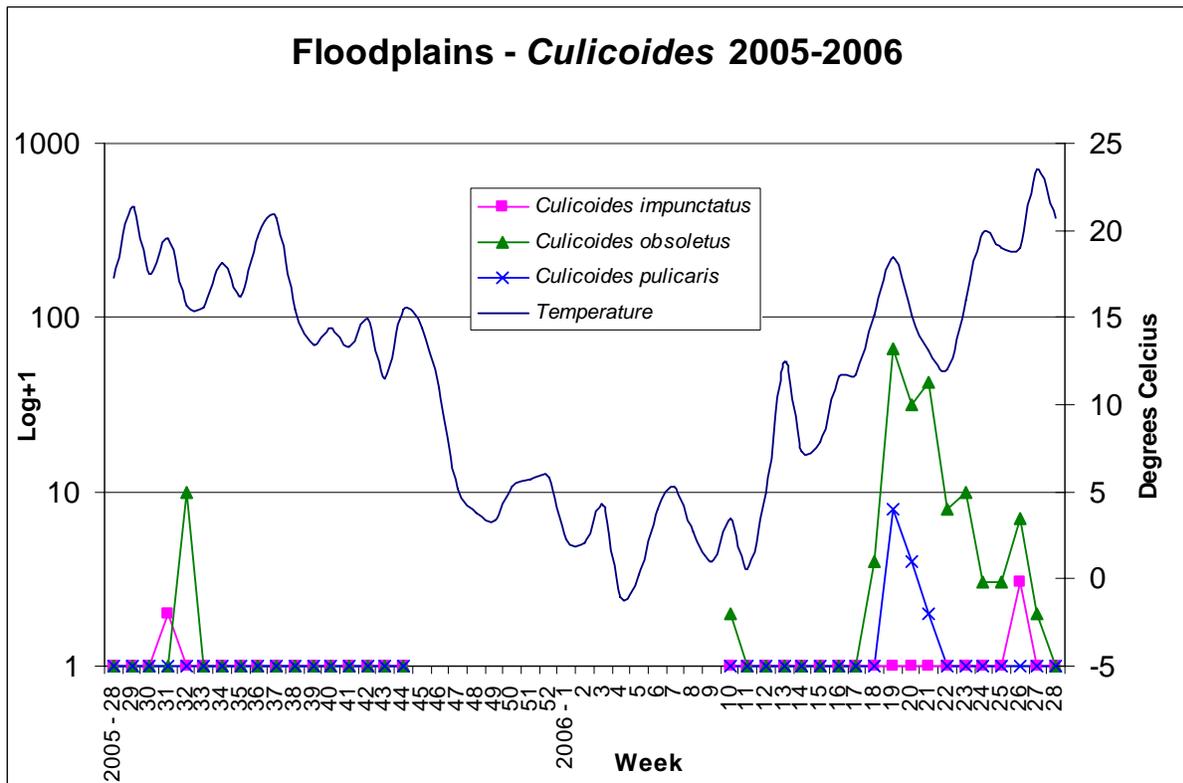
**Figure 5 Wetland area (Oostvaardersplassen): mean, maximum and minimum temperatures interpolated from weather stations.**

*Peat bogs and moores*

*Culicoides* species diversity was low compared to other habitats, but densities were higher. *Culicoides impunctatus* was by far the most common species. *C. obsoletus* and *C. pulicaris* were only present in small numbers **Figure 6**. Also between the peat areas the variation in collected numbers of *C. impunctatus* was large. Populations of *C. impunctatus* built up rapidly and can decrease until almost zero within a few weeks (Week 28-30 2005). Numbers of *C. impunctatus* in the Korenburgerveen in 2006 were much higher than in 2005, which further confirms the difficulty to predict the population dynamics of this species. As in the wetland area, midges appeared only at the beginning of May 2006, when especially the number of *C. impunctatus* rose quickly (**Figure 6**).



**Figure 6** Peat bogs and moores: catches of three most abundant *Culicoides* species in 2005-2006



**Figure 7** Floodplains: catches of three most abundant *Culicoides* species in 2005-2006

### Floodplains

*Culicoides* catches were relatively low in the floodplains compared to the other habitats. *Culicoides obsoletus* was the most common species and *C. impunctatus* was found only occasionally (**Figure 7**). Numbers were especially low in 2005. If this is due to flooding of the areas or a year effect is not clear. It is possible that *Culicoides* is nearly absent from the floodplains as larvae would drown during annual flooding. The floodplains are frequently flooded for a period of 7-14 days during high run-off of water from the river Rhine.

### Livestock farms

The most common *Culicoides* species at the livestock farms were species from the subgenus Avaritia, notably the taxa *C. obsoletus/C. scoticus*, *C. dewulfi* and *C. chiopterus*. In contrast with *Culicoides impunctatus*, which was common in other areas but not found throughout the whole sampling period, catches of the said taxa were relatively stable throughout the whole sampling period and the species was still present in week 44 (1st November) of 2005 (**Figure 8**). The data from the first weeks of the sampling period of 2006, show that there were no adults of *C. obsoletus* present or active during the winter. Because sampling did not continue during winter it is not possible to say for how long *C. obsoletus* was absent during winter and what the effect of temperature would be.

*C. obsoletus* was found in larger numbers at the livestock farms than at the other habitats and was found more often at the farms with a higher diversity of farm animals (Brömmels and Ravenswaard). Which kind of farm animals are indicative for the presence of *C. obsoletus* or which other factors influence its presence should be investigated in more detail. It is unclear why the number of *Culicoides* at the Ravenswaard in 2006 was four times higher then in 2005, or why the number of *Culicoides* in the Brömmels was twice as low in 2006 compared to 2005.

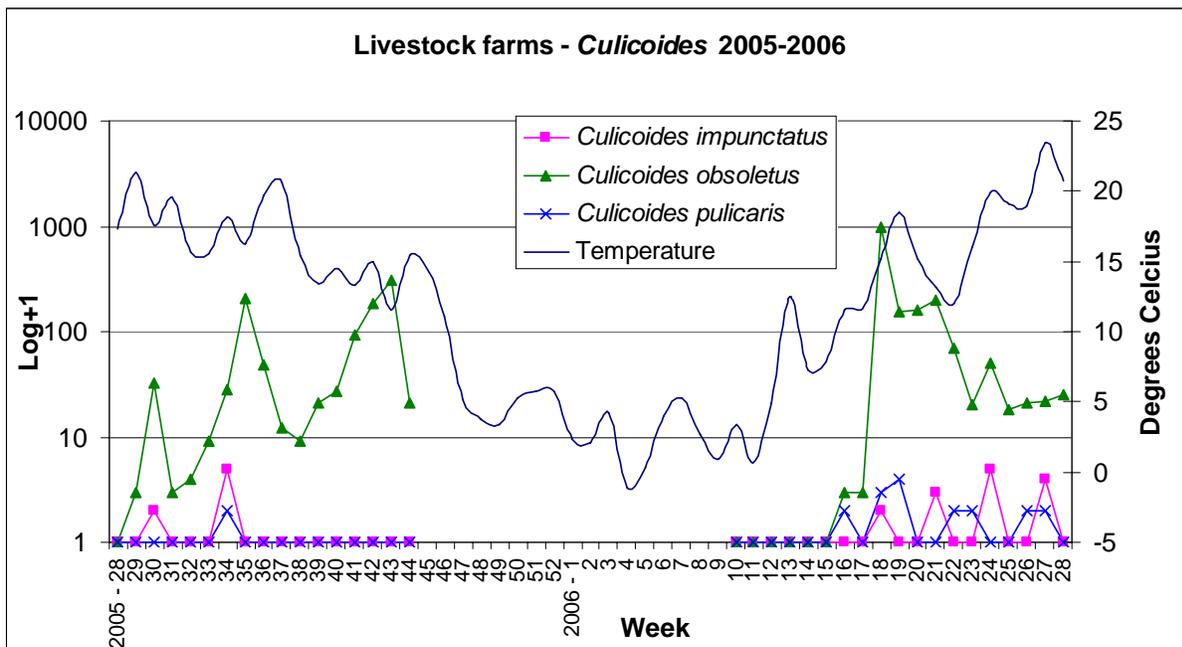
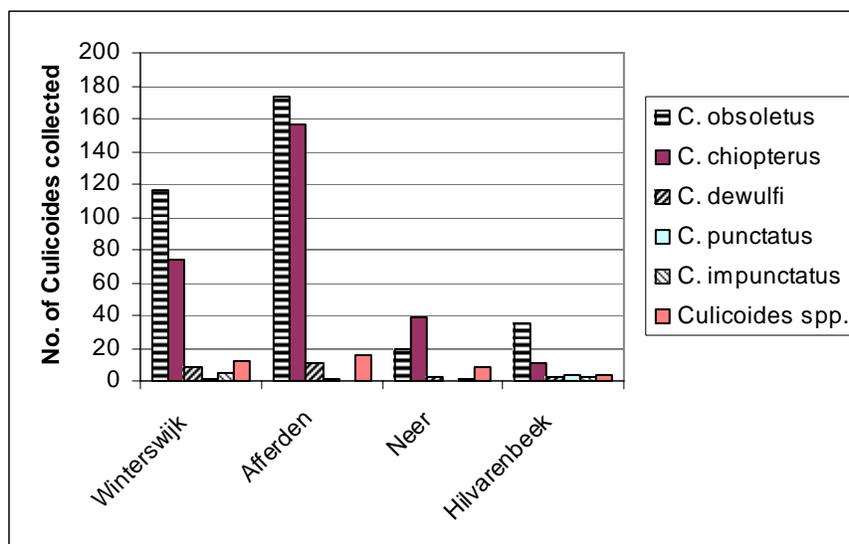


Figure 8 Livestock farms: catches of three most abundant *Culicoides* species in 2005 2006



**Figure 9** The species composition of *Culicoides* collected by CO<sub>2</sub>-baited traps on livestock farms, May-July 2005

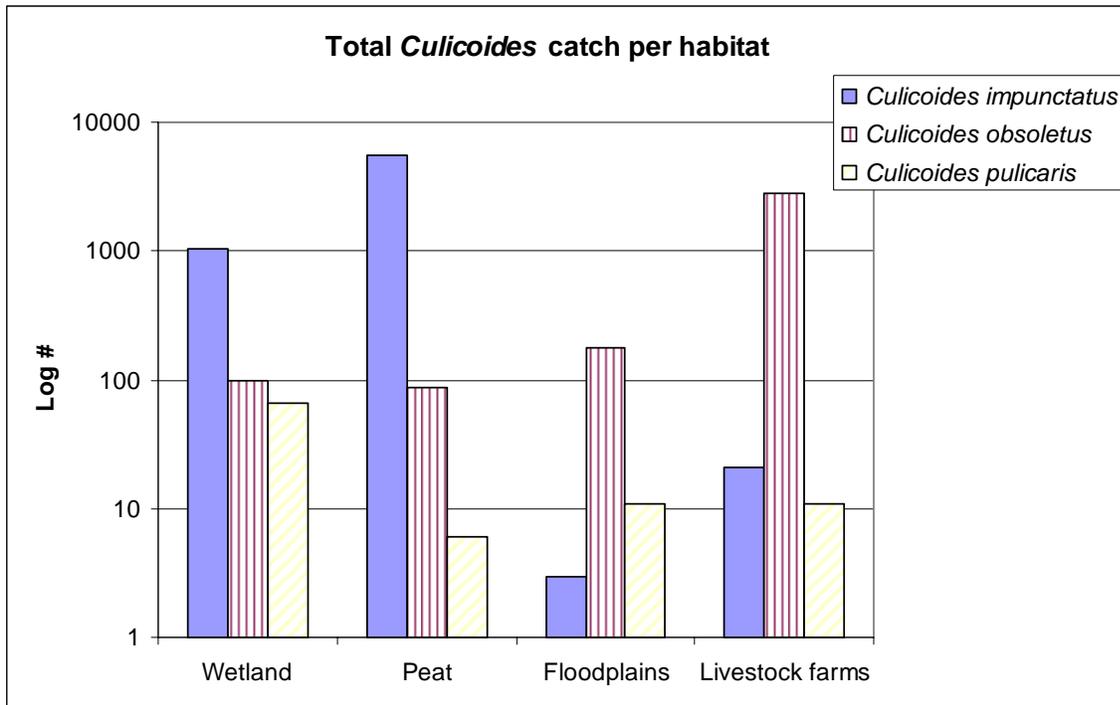
Because of the suspected role of *Culicoides* spp. in bluetongue virus transmission, in particular members of the subgenus Avaritia, biting midges were identified to sibling species as much as possible. The earliest date that we found *Culicoides* in 2006 on the farms was early May. Continuous monitoring since 1 March 2006 did not reveal any *Culicoides* spp. up to that time. Five species were identified (**Figure 9**). *Culicoides obsoletus* (which might have included *C. scoticus*) and *C. chiopterus* were the most common species of the *C. obsoletus* taxon, notably in Winterswijk and Afferden. *C. dewulfi*, in which BTV was isolated in September 2006, represented 2.86 – 3.39% of the catches. Most species were more abundant between May and 15 June, then in the period 15 June – 31 July.

Of particular note is the activity of *C. obsoletus* in the fall of 2005 (**Figure 8**): this group of *Culicoides* spp. remained active up to week 44, when our surveillance stopped. It would have been interesting to know for how many weeks this sibling group remained active. Our traps were standing outdoors, where *Culicoides* spp. are not expected to be found during low temperatures. Therefore, should any biting midge be caught in the odour-baited trap outdoors, we may expect greater activity in sheltered sites such as stables or walk-in sheds.

#### *Culicoides* abundance and habitats

As mentioned in the paragraphs before, vector abundance and species diversity varied much between the habitats.

*Culicoides* species were present in all habitat types, but with different composition of abundance: *Culicoides impunctatus* was found more often in the peat and wetland areas, *Culicoides obsoletus* on the other hand, was found more often on the livestock farms (**Figure 10**). As we have shown in the previous paragraphs, *C. obsoletus* on livestock farms consisted of a rich complex of species, dominated by *C. obsoletus* and *C. chiopterus*. In 2006 in all habitats *Culicoides* spp. became active only by the end of April.



**Figure 10** Total *Culicoides* catch per habitat of the three most common *Culicoides* species

## Discussion

This study demonstrates that many *Culicoides* species were present in the 4 different habitats across the country, although we caught them usually in relatively low numbers. Because no estimate (by mark-release-recapture studies) of the absolute density of midges was made, we do not know what the real density of biting *Culicoides* was. It became obvious that *C. obsoletus* and *C. pulicaris* were strongly associated with livestock farms. The former species has been incriminated as a vector of BTV in the 2006 epidemic. At least three members of the subgenus *Avaritia* have been found on the livestock farms: *C. obsoletus* s.s., *C. dewulfi* and *C. chiopterus* (**Figure 9**). However, the proportion of *C. dewulfi* in these collections was approximately 3%, and much lower than that of *C. obsoletus*. This suggests that in future the ecology of *C. obsoletus* should be investigated, to obtain information on biting behaviour, time of biting and winter phenology. *C. dewulfi* breeding has been associated with animal dung around stables and feeding troughs, but we know little about breeding habitats of other members of the subgenus *Avaritia* in north western Europe. Meanwhile, a short but intense surveillance of *Culicoides* species in Limburg in September 2006, using the Onderstepoort light trap, revealed again that *C. obsoletus* was the dominant species group among all *Culicoides* spp found (Takken et al. unpublished data and Meiswinkel et al. this volume). More than 88% of all collections consisted of members of this sibling group, including *C. obsoletus* s.s. and *C. dewulfi*. Nine percent consisted of *C. pulicaris*, another suspect species as BT vector.

It was interesting that of the four habitat types investigated, the livestock farms had the highest proportion of *C. obsoletus* and *C. pulicaris*. Both species are vectors of BTV in Italy and suspected vectors on the Balkan (De Liberato et al. 2005). Although these species were not expected to be active after the first night with frost in October, in November 2005 we found *C.*

*obsoletus* still active in one of the farms, suggesting that blood feeding was still occurring so late in the season.

*Culicoides impunctatus* was found most abundantly in the Oostvaardersplassen. It was also frequently collected in the peat bogs. This species is highly dominant in Scotland and Ireland, and has been incriminated a potential BTV vector. We have no evidence that it was one in The Netherlands. It was remarkable that this species was completely absent from Limburg province, approx. 80 km south of National Park de Grote Peel, an area where the species was quite abundant. It is possible that the collections in Limburg in 2006 were done too late for this species to still be present, because in the 2005 the species disappeared from our catches at the end of August (week 37).

There was no clear association between temperature and *Culicoides* activity. The spring of 2006 was relatively cold (**Figure 4**), and the first midges appeared only in the last week of April, in few numbers. In 2005, numbers of all *Culicoides* spp. fell sharply at the end of August. Although there was a markedly warm period in September 2005, this was not followed by a temporary rise in *Culicoides* numbers (**Figure 2**). By the end of May there were clear peaks of *C. impunctatus* and *C. obsoletus*, which fell sharply at the beginning of July.

One aspect of *Culicoides* behaviour, that is of relevance for the recent bluetongue epidemic in north western Europe, is the biting activity of the species that are indigenous to The Netherlands. We have frequently observed high biting intensity, evidenced by intense irritation by the investigators, during daylight hours, particularly on cloudy days. This behaviour, for instance, is well known from *Culicoides impunctatus*, and we suspect it also to occur in *C. obsoletus* and *C. pulicaris*. Thus, it is possible that biting by *Culicoides obsoletus* s.l. and *C. pulicaris* s.l. is not limited to nocturnal hours at this latitude.

We have used the Liberty Plus carbon dioxide baited trap as our main sampling tool for *Culicoides* and mosquito species. Whereas the sampling efficiency of the Liberty Plus trap for European *Culicoides* species is not known, in Scotland it has proven a highly effective trap for the sampling of *Culicoides impunctatus* (Mands et al., 2004). A rough comparison of this trap with data from the Onderstepoort light trap (Venter and Meiswinkel 1994) shows that the latter collects more *Culicoides* specimens than the carbon dioxide baited trap. The carbon dioxide baited trap, however, can operate 24 h per day, thus also catching those midges that are active during daylight hours. This might be a considerable advantage, because the indigenous *Culicoides* species in The Netherlands are not restricted to nocturnal activity as we experienced during our studies.

Another factor to consider is that in our study the traps were placed some distance away from buildings, in order to be in a more natural area. Thus, on livestock farms the traps were placed >50 m away from the nearest building. This may not have provided the best opportunity to collect the taxa *C. obsoletus/C. scoticus* and *C. dewulfi*, which are often found in proximity of stables and livestock herds.

The study demonstrates that *Culicoides* species are widely present in The Netherlands, and that several suspected BTV vectors appear to be particularly associated with livestock farms. With respect to the 2006 outbreak of bluetongue in The Netherlands, Belgium, Luxemburg, Germany and France, detailed studies on the ecology of indigenous *Culicoides* spp. is necessary, in order to

understand their diurnal rhythm, feeding and oviposition behaviour, as well as how this important group of vectors overwinters in the lowlands.

## **Acknowledgements**

The authors are indebted to the owners of the farms and the rangers of the nature reserves in which much of this study was conducted. We thank Aldo Bergsma for his assistance with geoinformation and data production. We thank Rudy Meiswinkel and Maria Goffredo for their discussions on current taxonomy of the *Avaritia* subgenus. This work was part of a grant provided by the Ministry of Agriculture, Nature and Food Quality under number TRC2005/2867.

## References

Blackwell A, Lock KA, Marshall B, Boag B and Gordon SC 1999. The spatial distribution of larvae of *Culicoides impunctatus* biting midges. *Medical and Veterinary Entomology* 13: 362-371.

Carpenter S, Lunt HL, Arav D, Venter GJ and Mellor PS 2006. Oral susceptibility to bluetongue virus of *Culicoides* (Diptera : Ceratopogonidae) from the United Kingdom. *Journal of medical entomology* 43: 73-78.

De Liberato C, Scavia G, Lorenzetti R, Scaramozzino P, Amaddeo D, Cardeti G, Scicluna M, Ferrari G and Autorino GL 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *Vet Rec* 156: 301-304.

Gomulski LM, Meiswinkel R, Delecolle JC, Goffredo M and Gasperi G 2005. Phylogenetic relationships of the subgenus *Avaritia* Fox, 1955 including *Culicoides obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2 ribosomal DNA sequences. *Systematic Entomology* 30: 619-631.

Kline DL 2002. Evaluation of various models of propane-powered mosquito traps. *Journal of Vector Ecology* 27: 1-7.

Mands V, Kline DL and Blackwell A 2004. *Culicoides* midge trap enhancement with animal odour baits in Scotland. *Med Vet Entomol* 18: 336-342.

Meiswinkel R, Gomulski LM, Delecolle JC, Goffredo M and Gasperi G, 2004. The taxonomy of *Culicoides* vector complexes - unfinished business. *Vet. Ital.* 40: 151-159.

Purse BV, Tatem AJ, Caracappa S, Rogers DJ, Mellor PS, Baylis M and Torina A 2004. Modelling the distributions of *Culicoides* bluetongue virus vectors in Sicily in relation to satellite-derived climate variables. *Med Vet Entomol* 18: 90-101.

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC and Baylis M 2005. Climate change and the recent emergence of bluetongue in Europe. *Nature reviews microbiology* 3: 171-181.

Venter GJ and Meiswinkel R 1994. The virtual absence of *Culicoides imicola* (Diptera: Ceratopogonidae) in a light-trap survey of the colder, high-lying area of the eastern Orange Free State, South Africa, and implications for the transmission of arboviruses. *Onderstepoort J Vet Res* 61: 327-340.

Verdonschot PFM 1996. Steekmuggen (Culicidae) in de Engbertsdijksvenen Monitoring van veensteekmuggen in 1995.

Weeda EJ 1981. Fuels for flight of the Colorado potato beetle. *Dissertatie LH Wageningen*, pp 1-141.

Weeda EJ, Schaminée JHJ and Van Duuren L 2005. *Atlas van Plantengemeenschappen in Nederland* (4 delen). KNNV Uitgeverij (gezamenlijk met Centraal Bureau voor de Statistiek,

Alterra, EWIN, Ministerie van Landbouw, Natuurbeheer en Voedselkwaliteit, Rijkswaterstaat en KIWA) Utrecht.

## **Annex B to Appendix 9**

**The *Culicoides* ‘snapshot’: a novel approach used to widely and rapidly assess vector densities during the 2006 outbreak of bluetongue (BT) in The Netherlands.**

*R. Meiswinkel*<sup>1</sup>

<sup>1</sup>CIDC-Lelystad, The Netherlands

## Summary

A novel method was developed and implemented in The Netherlands during the recent outbreak of bluetongue (BT) disease in sheep and cattle to obtain rapidly a ‘snapshot’ of *Culicoides* vector densities at national level. The country was divided into 110 raster cells each measuring 20 km x 20 km; within each cell a farm was selected with a minimum of 10 cattle and sampled for *Culicoides* for one night only using the Onderstepoort-type blacklight trap. Prior to deployment of the light traps in the field local veterinarians were trained in their use and in the preservation of captured *Culicoides*. The collections were despatched daily by courier to a field laboratory where the *Culicoides* were counted and identified. The ‘snapshot’ commenced 12 September 2006 and was completed 28 September; this coincided with the 5<sup>th</sup>-7<sup>th</sup> weeks of bluetongue virus (BTV) activity in The Netherlands when the number of weekly cases of disease was on the rise. Analysis of the 108 collections was completed on 5 October. An index of vector prevalence was developed based on the number of grid cells in which a taxon occurred and is represented by the formula:  $20^2\text{gPR}$  (= 20x20 km grid Prevalence Rate). The results from the ‘snapshot’ were as follows:

- a total of >34 000 *Culicoides* was captured ( $\mu$  = 322 midges/light trap) representing 16 species;
- not a single specimen of *C. imicola*, the principal Afro-Asiatic vector of BTV in southern Europe, was captured;
- the three European species of *Culicoides* implicated as vectors in the Mediterranean region and the Balkans occur also in The Netherlands;
- the Obsoletus Complex (represented by both vectors *C. obsoletus* and *C. scoticus*) was the most prevalent taxon ( $20^2\text{gPR}$ : 93.4%) followed by the Pulicaris Complex (77.4%);
- the Pulicaris Complex comprised at least six species. One of these, *C. pulicaris*, a proven vector in southern Europe, had a low  $20^2\text{gPR}$  of 14.2% indicating it to have played a very limited role in the outbreak;
- the next-most prevalent taxa were *C. dewulfi* (71.7%) and *C. chiopterus* (68.9%) both of which breed in cattle dung. This close association with livestock raises their potential as vectors of BTV;
- during the ‘snapshot’ overall numbers of *Culicoides* fluctuated >50-fold in synchrony with warming and cooling trends but would rebound rapidly indicating more intense cold weather is required before heightened mortalities are induced.

Increased ambient temperatures influence all life history traits of vector insects and accelerate development in the disease pathogens transmitted. The ‘snapshot’ provides veterinary authorities with baseline information around which to plan a sound vector surveillance strategy for the 2007 season — predicted to become the hottest on record. In highlighting areas of considerable vector density in The Netherlands, and thus at greatest risk to future incursions of BT, the ‘snapshot’ data will help also guide research on the ecological factors promoting risk, and if man-made, might be amenable to control. It is stressed that a vector surveillance program must be built on a firm taxonomic base because misidentifications can fatally flaw the mapped seasonal and geographic distribution patterns upon which veterinary authorities depend.

## Introduction and Objectives

In August 2006 bluetongue disease in sheep and cattle was detected for the first time in central Europe. It was first identified in sheep in the village of Kerkrade in the province of Limburg, south-eastern Netherlands (Vrouenraets 2006). Over the ensuing four months it spread into western Germany, across much of Belgium and on into northern France and Luxembourg covering an estimated 240 000km<sup>2</sup> of territory. Both in Germany and in The Netherlands it reached almost to latitude 53°N, which is the furthest north BTV has penetrated anywhere in the world. It is assumed that the virus arrived in one or other infected animal but all avenues of enquiry have failed to identify both the port of entry and the exact location of the index case. The extensive speculation around its mode of entry includes also the postulate that infected specimens of the principal Afro-Asiatic vector of BT i.e. *C. imicola* entered the region on an aeroplane. Whatever its route of entry the virus was isolated by the Central Reference Laboratory (Pirbright, England) of the European Community and identified as bluetongue virus serotype 8 (BTV-8). Phyletic analyses have shown it to be most closely related to a 1982 strain of BTV-8 from West Africa. The strain is not derived from a polyvalent live-attenuated vaccine used some years previously in parts of the Mediterranean Basin.

The BTV is comprised of 24 serotypes; these are distributed throughout the tropics and subtropics of the world. The most serotypes are to be found in Africa and in India ( $\approx$  20 each). Each affected region of the world has a specific number of serotypes and because they are more or less stable in their occurrence has led to them being divided amongst five world episystems (Tabachnick 2004). The Mediterranean episystem comprises 12 serotypes; normally one or other of these will emerge (mostly from the east) and cyclically, at 20- to 30-year intervals, spread westwards. But since 1998 this pattern appears broken because multiple serotypes of BT have swept across the Basin in successive years and waves. In the process the disease has affected at least 15 countries a number for the first time; also, it has progressed further north than ever before causing tremendous losses both in terms of the number of sheep lost (close to one million) and through widespread blockages in live animal trade.

BTV is transmitted by biting midges of the genus *Culicoides*; approximately 30 species are now known to be involved in its transmission in various parts of the world. These are listed elsewhere according to subgenus and species complex (Meiswinkel et al. 2004). In southern Europe at least four species have been implicated. The most important of these, and accounting for at least 90% of BTV transmission, is the Afro-Asiatic *C. imicola*; the secondary vectors, and ones endemic to Europe, are *C. obsoletus*, *C. scoticus* and *C. pulicaris*. Only in 1982 was *C. imicola* discovered to occur along the northern margin of the Mediterranean Basin; perhaps for this reason some workers believe it to be a recent invader (Purse et al 2005) and that, under the influence of a warming global climate, it will move northwards into Europe posing an ever-increasing threat to livestock there. An alternate possibility is that *C. imicola* has long been established in the region but has simply gone undetected due to a paucity of entomological surveys and the use of insensitive tools. In this scenario the northward movement of BT (and other *Culicoides*-borne orbiviruses) will likely occur via multiple vectors and that these — in the main — will constitute species endemic to Europe.

Stated broadly the extant knowledge on the *Culicoides* of central Europe remains inadequate. Whilst fairly comprehensive species checklists exist for most countries there is a gap in information on the seasonal and geographic distributions of vector *Culicoides*. This lack has

crippled somewhat the ability of countries affected by BT to respond rationally to the recent and quite sudden advent of the disease. Not knowing where and when the vectors occur means that areas at lower risk, or vector-free areas suitable for quarantining and for export purposes, cannot be identified and so utilised for the maintenance of trade. Conversely, areas with high vector densities also remain unidentified; these represent foci of risk and must be pinpointed because they could, in part, be man-made and thus amenable to control.

The ‘snapshot’ has been developed gradually over a number of years as a method for rapidly assessing the status of *Culicoides* vector populations at the national level. Depending on the size of the country the ‘snapshot’ can be completed in a matter of weeks and so provide the veterinary authorities with solid baseline information on which to plan future surveillance activities. It is usually implemented during periods of outbreaks of disease and thus provides an instantaneous overview of the vector situation both in terms of the species and the numbers of *Culicoides* active. Until now the primary emphasis of such surveys has been to establish whether the principal vector of BT in the Old World i.e. *C. imicola* is present within an infected area. But since 2003 the extensive outbreaks of BT across the Mediterranean region and the Balkans has revealed that a further three species of *Culicoides* are involved in the epidemiology of the disease. This advent of additional vectors is viewed with some concern as it is well established that increased ambient temperatures raise the potential of arthropod vectors to transmit pathogens (LaPointe, Benning and Atkinson 2005), which implies that global warming may be ‘awakening’ a latent vectorial potential in still others of the >120 species of *Culicoides* known to occur in western Europe. The ‘snapshot’ is thus able to provide information rapidly on the species active within a given area and what their abundances are around vulnerable livestock. In undertaking ‘snapshot’ surveys it is important they be underpinned by a sound taxonomy of the *Culicoides* of Europe. This is because misidentifications will fatally flaw the resulting vector distribution maps upon which veterinary authorities depend when developing safe animal movement trade protocols and defining areas of low-risk. The taxonomy of the *Culicoides* of Europe presents particular difficulties especially in regard to vectors; an example of the problem is used for brief discussion. Finally, a specific shortcoming of the ‘snapshot’ — indeed of all light trap-dependent surveys — is that the light trap is only able to monitor *Culicoides* that are nocturnally active and unable to attract and capture those which are also diurnally active. The need to quantify diurnal abundances and livestock attack rates is discussed as these have implications for the transmission of BT.

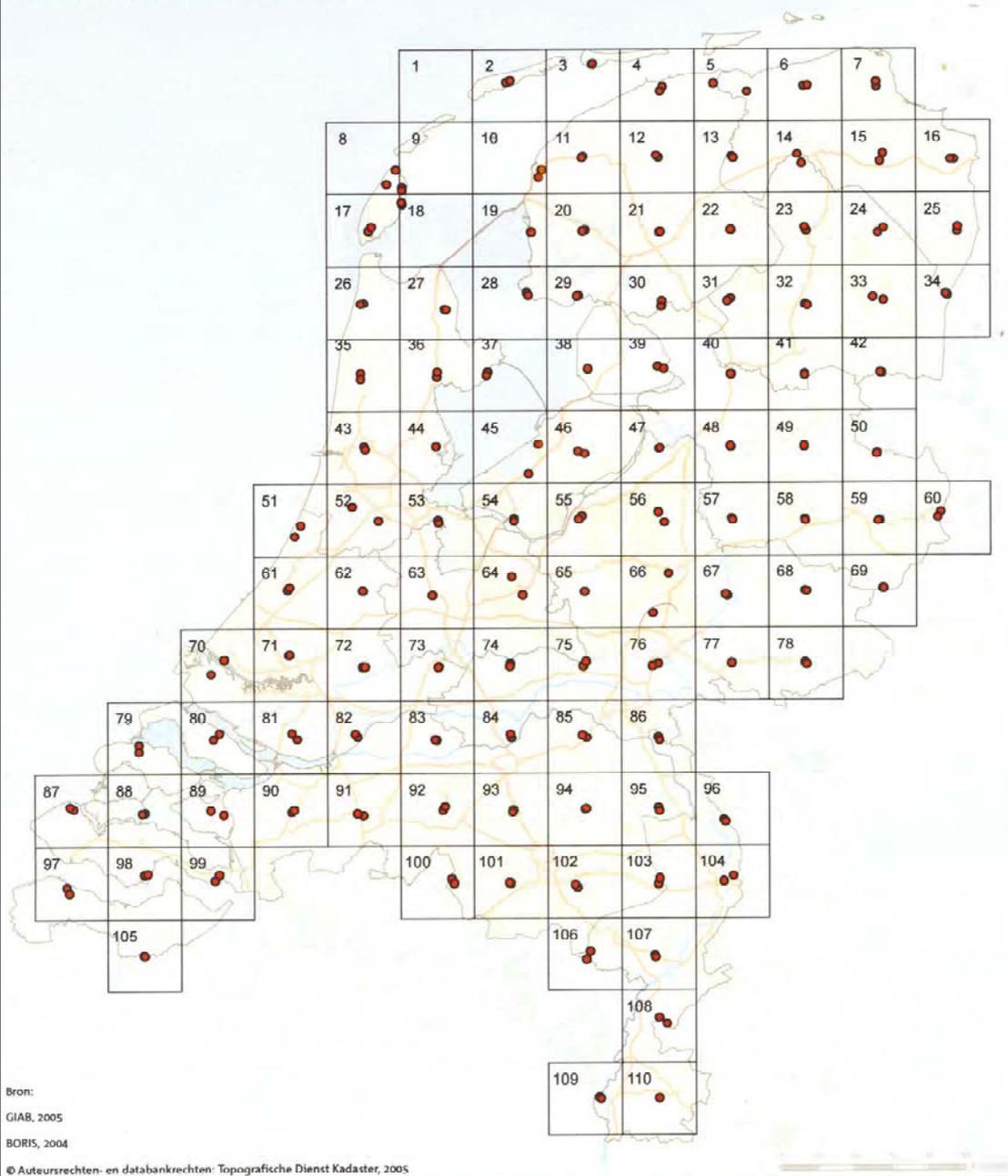
## Materials and Methods

For the ‘snapshot’ The Netherlands was divided into a raster grid of 110 squares each measuring 20 km x 20 km (Fig. 1); in each grid a farm with a minimum of 10 cattle was selected on which a single *Culicoides* light trap collection was to be made. Five teams of local veterinarians were convened in Den Haag and trained for three hours in the choice of trapping sites, use of the traps and in the handling of captured insects. Each team was issued with the entire collection kit as described in Goffedo and Meiswinkel 2004; Onderstepoort 220v blacklight traps were used and the collections made in accordance to the protocols of the Italian Reference Centre for Exotic Diseases (CESME, Istituto Zooprofilattico Sperimentale dell’Abruzzo e Molise “G. Caporale”). A total of nine traps were deployed and moved daily until all grids had been sampled; it was planned to complete the field survey in two weeks. The light trap collections were sent by courier daily to a field laboratory situated in the south-eastern province of Limburg where they were

immediately analysed. The analyses were completed on 5 October 2006. The total number of insects, the total number of *Culicoides* and the total of each species (or species complex) was determined for each catch. The results are tabulated and the distribution of 16 species of *Culicoides* mapped. The maps are colour-coded according to the log numbers of each species collected in each grid. The grid Prevalence Rate (gPR) is the percentage of grid cells (out of a possible 108) in which a taxon occurs; because sampling was done in 20 km x 20 km-sized grids we refer to a taxon's grid prevalence rate as 20<sup>2</sup>gPR. *Culicoides* were identified using the keys of Campbell and Pelham-Clinton, 1960 and Delécolle, 1985 and augmented by data gleaned during two molecular investigations on the *Obsoletus* and *Pulicaris* vector complexes in Italy (Meiswinkel et al. 2004; Gomułski et al. 2005, 2006).

# Overzichtskaart met monitoringsgebieden

Rundveebedrijven met 10 of meer runderen



Bron:

GIAB, 2005

BORIS, 2004

© Auteursrechten- en databankrechten: Topografische Dienst Kadaster, 2005

### Legenda

- Bebouwde kom
- Monitoringsgebieden (20x20km)
- Grotere wateren
- Snelwegen
- Bos
- Rivieren
- Provinciegrens
- Rundveebedrijven (= >10 runderen)



Ministerie van Landbouw,  
Natuur en Voedselkwaliteit  
GIS COMPETENCE CENTER

Opdrachtgever: Dienst Regelingen  
 Project: Blue Tongue 2006  
 Onderwerp: Overzichtskaart met rundveebedrijven (>10) in monitoringsgebieden  
 Status: Definitief  
 Datum: 08/09/2006 Paraaf: \_\_\_\_\_  
 Schaal: 0.1  
 Projectnr.: GCCA0022\_CRISIS\_EN GIS/Uitbraak\_BT  
 GISprojectnaam: Overzichtskaart\_monitoringsgebieden.mxd

Figure 3. The Netherlands divided into 110 raster cells each measuring 20 km x 20 km and sampled for *Culicoides* for one night only using an Onderstepoort-type blacklight trap (12-28 September 2006).

## Results

The 'snapshot' gave the following results:

- a total of 108 light trap collections yielded 386 754 insects;
- of these 34 152 (8.83%) were *Culicoides* representing 16 species;
- the numbers of *Culicoides* captured ranged between 0-8 722 ( $\mu = 322.0$ );
- in only eight collections were >1 000 *Culicoides* captured;
- *Culicoides* were absent from only 3 collections;
- the most widely distributed taxon was the Obsoletus Complex with a 20<sup>2</sup>gPR of 93.4% and represented by both *C. obsoletus* and *C. scoticus*;
- the second-most prevalent taxon was the Pulicaris Complex (20<sup>2</sup>gPR: 77.4%);
- followed by *C. dewulfi* (71.2%), *C. chiopterus* (68.9%) and the Nubeculosus Complex (28.3%);
- not a single specimen of *C. imicola*, the principal vector of BT in southern Europe, was captured;
- the average numbers of *Culicoides* captured in 7-9 light trap collections made daily ranged between 29.2-1 677.5. This wide fluctuation over 12 sampling nights, and which included a >50-fold decline between 15 and 20 September, occurred synchronously across The Netherlands in response to brief and separate periods of inclement weather;
- All three vector taxa occurred abundantly in 11 (10%) of the grids sampled. These were distributed across the length of The Netherlands and represent areas of future risk.

The total abundances of the *Culicoides* in the 108 collections were as follows:

- the Pulicaris Complex was dominant (14 155 specimens; 41%);
- it comprised at least six species the most abundant being *C. punctatus* (10 445; 73.8%). The remaining taxa — in descending order of abundance — were *C. newsteadi sensu* Delécolle 1985 (2 869; 20.3%), *C. halophilus* (331; 2.34%), *C. pulicaris* (290; 2.05%), *C. lupicaris* (116; 0.82%) and *C. impunctatus* (1; 0.007%);
- the second-most abundant taxon was the Obsoletus Complex (12 448; 36%);
- it comprised at least two species (*C. obsoletus* and *C. scoticus*). Based on the number of males captured, the ratio of the former to the latter was 7:1. Whether the same ratio can be applied to the more abundant female sex is not known;
- the total numbers of *C. dewulfi* and *C. chiopterus* were respectively 3 863 (11.31%) and 2 507 (7.34%);
- the remaining six species of *Culicoides* amounted to 1179 specimens (3.45%); approximately half of these belonged to the Nubeculosus Complex.

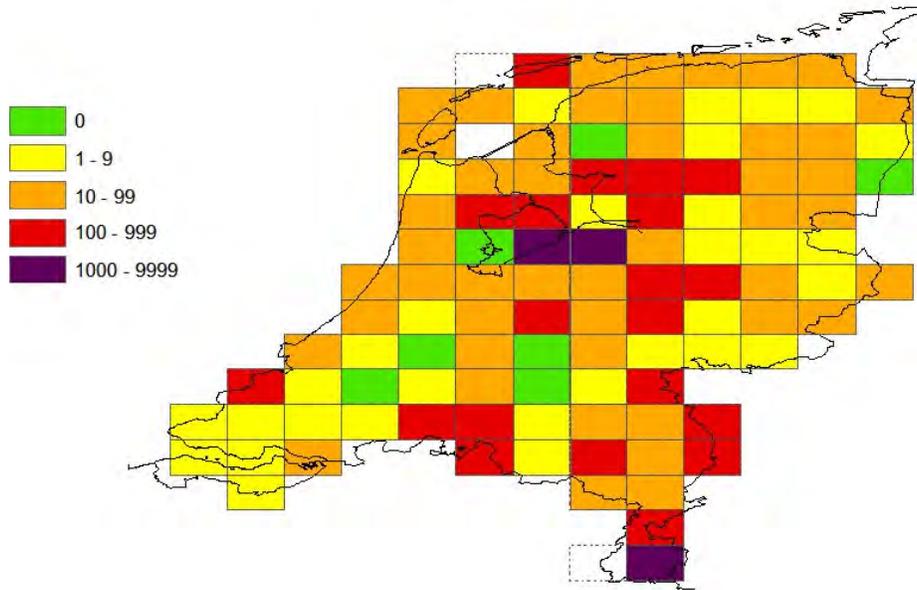
The 'snapshot' maps show that:

### The Obsoletus Complex

was the most widespread taxon in the Netherlands (**Figure 2**) with a 20<sup>2</sup>gPR of 93.4%. This may be due to at least one of the constituent species (*C. obsoletus*) breeding in forest leaf litter enabling it to penetrate wherever deciduous trees occur, which would include also urban environments. Because the Obsoletus Complex is a known vector of BT in southern Europe its widespread occurrence (and heightened abundances) place a significant portion of The Netherlands at risk.

**Figure 4**

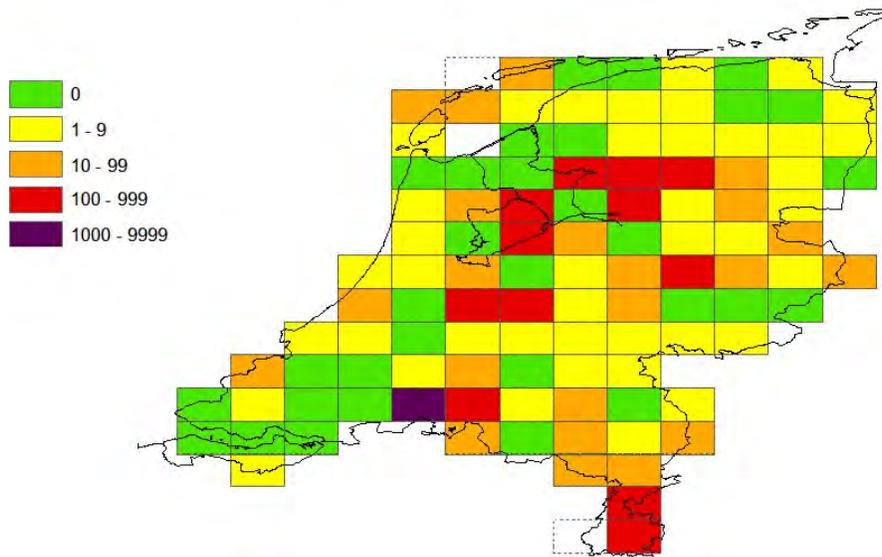
**Obsoletus Complex**



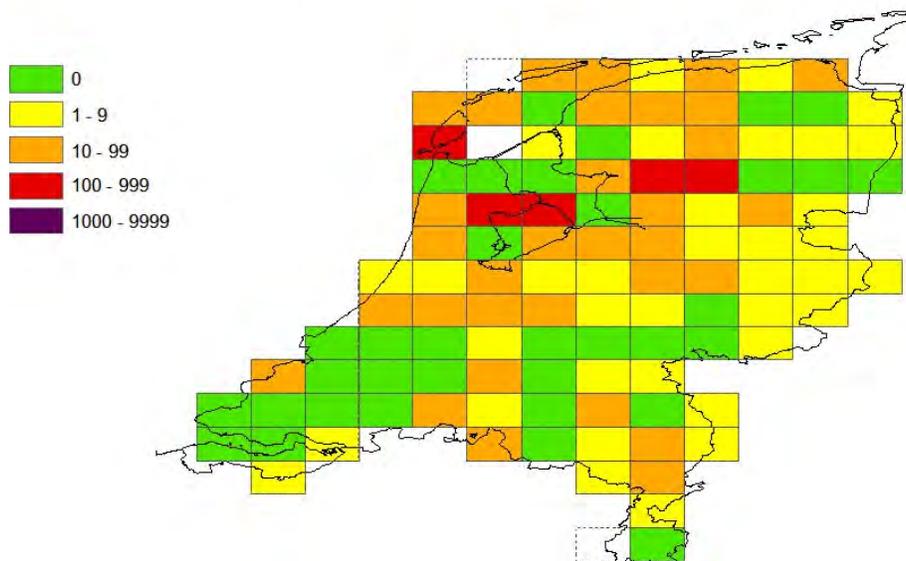
C. dewulfi and C. chiopterus

- *C. dewulfi* (Figure 3) and *C. chiopterus* (Figure 4) occur also widely across The Netherlands with respective 20<sup>2</sup>gPR's of 71.7% and 68.9%; this is not unexpected as they breed exclusively in the dung of cattle (and horses). During more intensive investigations in the region of Gulpen, Limburg province, *C. dewulfi* was implicated as a hitherto unrecognised potential vector of bluetongue. This important finding, made in conjunction with the CIDC, Lelystad, is discussed below.

**Figure 3** *C. dewulfi*



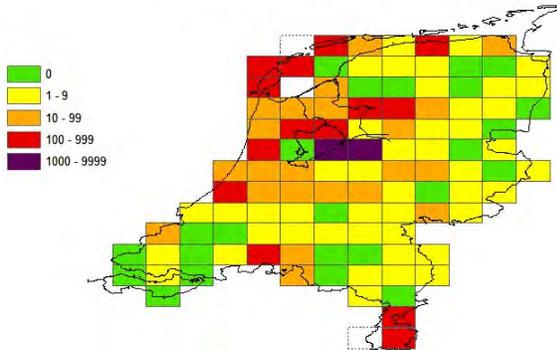
**Figure 4** *C. chiopterus*



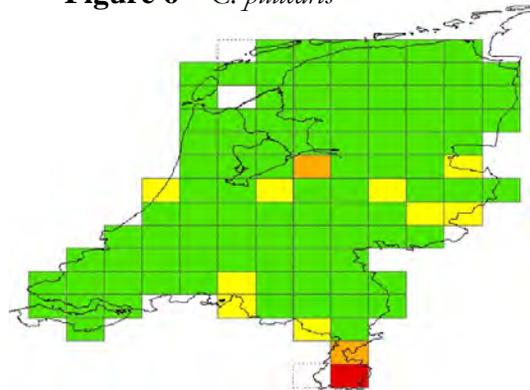
## The Pulicaris Complex

- occurred in 82 of the grids sampled (**Figure 5**) giving an overall 20<sup>2</sup>gPR of 77.4%. *C. pulicaris*, the only species of the Pulicaris Complex to be implicated in the transmission of BT in southern Europe, was not found abundantly and had a low 20<sup>2</sup>gPR of 14.2%. Although it was most prevalent in the south-east (Fig. 6) of the Netherlands — where the outbreak was at its most intense — the very low abundances of *C. pulicaris* suggest it played no vectorial role.

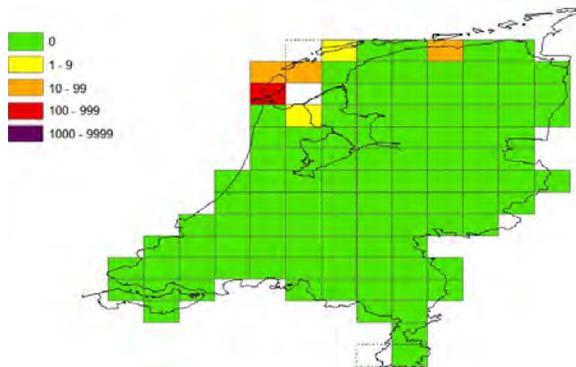
**Figure 5** Pulicaris Complex



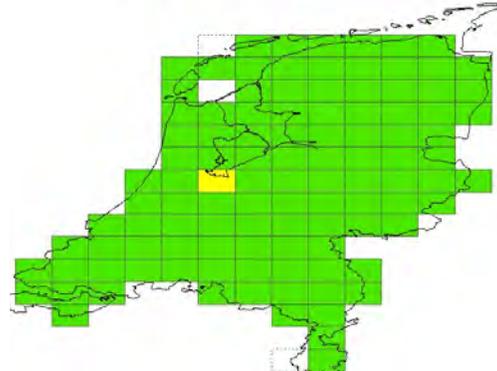
**Figure 6** *C. pulicaris*



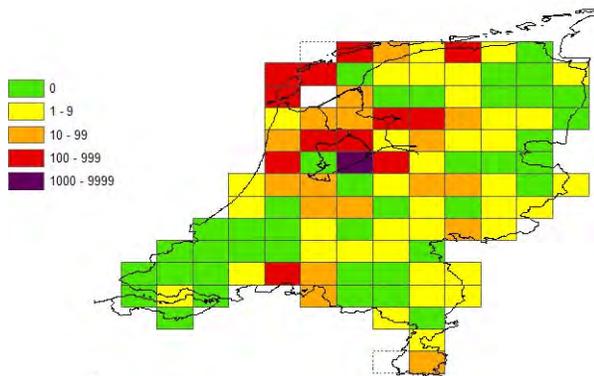
**Figure 7** *C. halophilus*



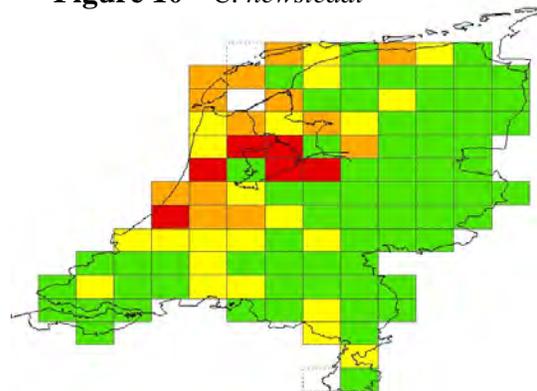
**Figure 8** *C. impunctatus*



**Figure 9** *C. punctatus*



**Figure 10** *C. newsteadi*



- only one (*C. punctatus*) of the six species of the Pulicaris Complex was widely distributed the remaining five being restricted to certain regions. This is due to specific breeding habitat preferences e.g. saline coastal areas for *C. halophilus* (Figure 7) and peat bogs for *C. impunctatus* (Figure 8).
- the most prevalent and widespread taxon of the Pulicaris Complex was *C. punctatus* (Figure 9) with a 20<sup>2</sup>gPR of 63.2%; however, it has never been implicated in the transmission of BT. The second-most prevalent taxon, with a 20<sup>2</sup>gPR of 38.7%, was *C. newsteadi sensu* Delécolle (1985); it has also not been implicated as a vector. In The Netherlands *C. newsteadi* had a distinctive north-west distribution (Fig. 10) far removed from the epicentre of the outbreak.

## Discussion

The *Culicoides* ‘snapshot’ opened a window on the vector situation in The Netherlands as it existed during the unprecedented outbreak of bluetongue disease that occurred in the latter half of 2006. It revealed that *Culicoides* occur throughout the length and breadth of the country and although only 16 species were captured they included three of the four species incriminated in previous years in the transmission of bluetongue (BT) in southern Europe. These are *C. obsoletus*, *C. scoticus* and *C. pulicaris*; in 11 of the 108 grids sampled all three vectors were found abundantly and thus represent areas of heightened risk. Not a single specimen of *C. imicola*, the principal vector of BT in southern Europe and in Africa, was captured.

### The Obsoletus Complex

Within the palaeartic region the Obsoletus Complex comprises six species; at least four of these occur in western Europe and includes the vectors *C. obsoletus* and *C. scoticus*. It has long been apparent that the former is the most widely distributed and abundant *Culicoides* to occur in the region; but in recent years it has emerged that its congener *C. scoticus* occurs also widely and in some locales has been found to be either equally or more abundant than *C. obsoletus*. Unfortunately, the females of these two species are impossible to identify reliably in the field; although the males can be identified easily they are captured rarely and so cannot be relied upon to determine relative species abundances — and seasonal and geographic distributions — accurately. Nevertheless, the males of both species were captured during the ‘snapshot’ and thus it is certain that both *C. obsoletus* and *C. scoticus* occur in The Netherlands. The Obsoletus Complex is undoubtedly the most-prevalent taxon but atlassing the distribution of its two constituent species will in future depend on the molecular identification of field-captured specimens.

*C. obsoletus* has been reported to breed in forest leaf litter (Dzhafarov, 1964) and its close association with deciduous forests in Italy demonstrated (Conte et al in press). Elsewhere in the palaeartic region *C. obsoletus* has been reported from habitats other than forest litter including horse dung and garden compost heaps (Campbell and Pelham-Clinton 1960). A similar situation prevails for its congener *C. scoticus*: it was originally bred from decaying fungi (Buxton, 1960) in the British Isles but was found subsequently to inhabit also marshy sites (Boorman and Goddard 1970). It remains to be demonstrated whether both taxa are particularly plastic in their choice of breeding habitat or whether this plasticity masks the sympatric occurrence of a number of closely related species.

## The Pulicaris Complex

The ‘snapshot’ showed the Pulicaris Complex to be — marginally — the most abundant taxon in The Netherlands. But despite being represented by at least six species it was less prevalent than the Obsoletus Complex. The clear majority (94.1%) of specimens of the Pulicaris Complex belonged to *C. punctatus* and *C. newsteadi*; the remaining four species were uncommon (*C. pulicaris* and *C. halophilus*) or rare (*C. lupicaris* and *C. impunctatus*). The two commonest species had a predominantly north-western distribution (Figs. 9 and 10) occurring some distance from the epicentre of the outbreak (which was concentrated in the south-east of the country and from whence it progressed only slowly, and only part of the way, northwards). It appears the Pulicaris Complex played little or no role in the outbreak of BT; however, this does not preclude it from becoming important in the future.

## Unresolved taxonomy of the Pulicaris Complex: a brief word

A brief word on the taxonomy of the Pulicaris Complex in Europe is necessary because in any vector surveillance program the misidentification of taxa will greatly reduce the quality of the information obtained. As a taxon the Pulicaris Complex is more correctly referred to as the subgenus *Culicoides* Latreille, 1809. But most authors interpret the taxon too broadly including almost 50 species; in its strict sense the subgenus contains only 12 species of which at least eight occur in western Europe. The taxonomy of the Pulicaris Complex is also in flux at the species level (Meiswinkel *et al.*, 2004; Gomulski *et al.*, 2006). For example the second-most abundant species in The Netherlands i.e. *C. newsteadi sensu* Delécolle, 1985 differs from *C. newsteadi sensu* Austen, 1921 (described originally from Palestine and which has a significantly darker wing pattern). In turn a dark wing is also the reason why most authors consider a third species, *C. halophilus* Kieffer 1924, to be a synonym of *C. newsteadi sensu* Austen. However, as made evident from the list (and maps) of species provided above, we consider *C. halophilus* to be a valid taxon and follow Edwards’ (1939) dictum that it is the northern (and distinct) ‘form’ of Austen’s southern *C. newsteadi*. Both are distinct from *C. newsteadi sensu* Delécolle 1985. But we remain unsure of its exact name and so will, in the interim, and as done by other researchers, refer to as *C. newsteadi sensu* Delécolle 1985. The principal point of this brief review is to emphasise two points: 1. the taxonomy of the *Culicoides* of Europe remains incomplete, and 2. this can compromise vector surveillance programs, which must be built upon a firm taxonomic foundation. Misidentifications can fatally flaw vector distribution maps used by veterinary authorities to develop livestock movement protocols and to define vector-free periods and zones.

## *C. dewulfi* and its biology

As described in detail in Annex 1 (p. 16) and as reported in ProMED-mail (Archive number 20061024.3042 of 24-Oct-2006) *C. dewulfi* was implicated in The Netherlands in October 2006 as a potential novel European vector of BTV. Although it remains to be more definitively implicated as a vector it would be prudent to document its spatial and temporal distribution in central Europe. Although described from neighbouring Belgium 70 years ago (Goetghebuer 1936), *C. dewulfi* has never been recorded before from The Netherlands. This is likely another result of misidentification because the female possesses a very vague and ill-defined wing pattern; because of this it is lumped mistakenly into the Obsoletus Complex. A recent molecular study based on ITS2 has confirmed *C. dewulfi* to not form part of this complex but to be a monophyletic taxon within the European fauna of the subgenus *Avaritia* (Gomulski *et al.* 2005).

The biology of *C. dewulfi* is unique and likely explains why it is the second-most prevalent taxon in The Netherlands. It breeds in cattle and horse dung (Kettle and Lawson 1952) ensuring it will be found almost wherever these animals occur; this inter-dependence between an insect and a host — and including one that is susceptible to BT — increases the vector potential of *C. dewulfi*. As shown by Townley et al (1984) *C. dewulfi* feeds also avidly on the horse, which brings it into play as the potential vector of still other orbiviral diseases, more specifically African horse sickness (AHS). Of added concern is that the biology of *C. dewulfi* mimics that of other vectors of BT known to occur elsewhere in the world i.e. *C. brevitarsis* and *C. wadai* in south-east Asia and Australia and *C. bolitinos* in sub-Saharan Africa. All of these vectors breed in cattle and buffalo dung; thus *C. dewulfi* belongs to a formidable quartet of world vectors breeding exclusively in animal dung. Of these only *C. dewulfi* is known to breed also in horse dung. In this context it should be noted that the third-most prevalent taxon captured in The Netherlands is *C. chiopterus*, which breeds also in cattle dung; however, it is not implicated in the transmission of BT.

### *Culicoides imicola*

The principal vector of BTV in southern Europe is the Afro-Asiatic *C. imicola* responsible for as much as 90% of the overall disease transmission rate. It is also a formidable vector of African horse sickness (AHS) and thus its presence in Europe is regarded with some trepidation. Its recent discovery in countries on the northern margin of the Mediterranean Basin has led to the view that it is a recent invader and may be spreading rapidly northwards into Europe (Purse et al. 2005). An alternate view is that *C. imicola* has long been resident in southern Europe and that its fragmented occurrence is due to a strong predilection for nutrient-rich and moisture-retentive clayey soils in flat terrain but which occur only patchily across the region. A predilection for certain soils — if demonstrated — may ultimately limit the capacity of *C. imicola* to move further northwards into Europe.

### The ‘snapshot’: its value as a sampling method

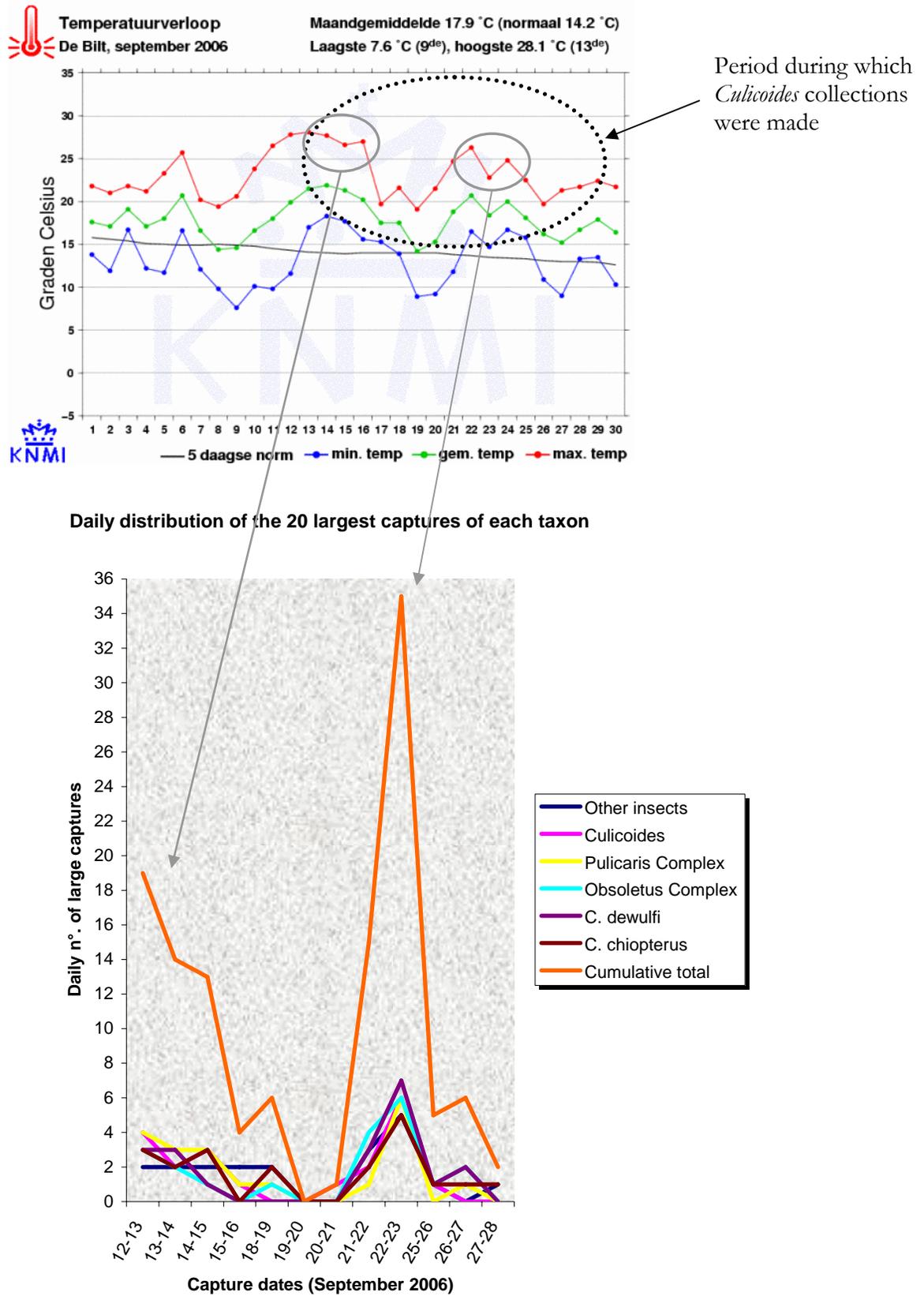
The ‘snapshot’ revealed rapidly that all species of *Culicoides* — excepting *C. imicola* — incriminated in the epidemiology of BTV in the Mediterranean region and the Balkans occur also in The Netherlands and that some, like *C. obsoletus*, occur abundantly and widely. The perception that vector distribution patterns alter from year to year thereby invalidating ‘snapshot’ surveys is contrary to findings made in Italy following the arrival of BT on Sardinia and Sicily in the year 2000 (Goffredo et al. 2001). The highly fragmented pattern of distribution of *C. imicola* revealed at the time, though unrefined, has proved highly reliable with no subsequent evidence for its oft-predicted dissemination into new areas. However, significant inter-annual changes in its abundance levels do occur the result of oscillations in local weather patterns. The ability of the insect vector to respond adaptively to such changes implies that one consequence of a warming global climate is that it may induce a lengthening in the breeding season of *Culicoides*. This will likely translate into quicker and more frequent blood feeding cycles, which will raise the vectorial potential of the species that feed on livestock. The ‘snapshot’ exposed also areas in which multiple vectors occur; these are at greatest risk to future incursions of BTV and should thus be investigated to identify the ecological factors favouring vector proliferation and to establish whether these are man-made and therefore amenable to control.

### The ‘snapshot’: its shortcomings as a sampling method

A significant shortcoming of the ‘snapshot’ — indeed of all light trap-dependent studies — is that diurnally active *Culicoides* are not attracted to a trap which has to compete against natural light. It is generally supposed that *Culicoides* are strictly nocturnal but a number of European species are also troublesome in the day and include *C. obsoletus*. Two day-biting peaks are widely reported: one after sunrise the other close to sunset. These hours of attack are lengthened when low-light, overcast conditions prevail and in forested environs can induce *Culicoides* to bite throughout the day; from vegetated shelter *C. obsoletus* will move out to bite pastured livestock (Dzhafarov, 1964). The intensity of such daytime attacks needs to be quantified because it has implications for the diurnal transmission of BTV; this is especially relevant for *C. obsoletus* and for species breeding in cattle dung, which are widely prevalent across The Netherlands.

### Fluctuating *Culicoides* abundances and varying temperatures

It has been demonstrated for a wide variety of arthropod-borne pathogens that increased ambient temperatures can widen the geographical range of a disease (LaPointe, Benning and Atkinson 2005). The 800 km northward ‘jump’ by BTV into central Europe in 2006 — one of the warmest years on record — would seem to illustrate this maxim perfectly. The ‘snapshot’ was conducted during a period when meteorological records show it to have been the warmest September since records began in 1706 exactly 300 years ago. Despite such generally favourable conditions 75% of the *Culicoides* were captured only on the warmest nights (12-15, 21-23 September); the average numbers of *Culicoides* captured in the 7-9 light trap collections made daily ranged between 29.2-1 677.5. This wide fluctuation in *Culicoides* activity over 12 sampling nights, occurred in synchrony across The Netherlands and appears correlated with rising and falling temperatures (Fig. 11). Thus brief periods of inclement weather induced a 50-fold decline in *Culicoides* numbers but these would rebound rapidly when warmer conditions returned; this indicates that a more severe cooling of the weather is required before heightened *Culicoides* mortalities occur. The data show also that all the taxa responded similarly to these fluctuations in the weather pattern. Since the spread of BTV-8 across more than 240 000 km<sup>2</sup> of central Europe did not require simultaneous invasion by a tropical vector it warns us that still other species of temperate *Culicoides* may be equally effective at replicating BTV under rising average global temperatures. For this reason it is essential that the veterinary authorities in central Europe (and further northwards) embark on comprehensive vector surveillance programs to establish exactly which species of *Culicoides* become abundant in the vicinity of livestock.



**Figure 11. Correlation between temperature and *Culicoides* abundances in The Netherlands (12-28 September 2006)**

## Annex 1. *Culicoides dewulfi*: a novel potential vector of BTV from central Europe

R. Meiswinkel<sup>1</sup>, A. Conte<sup>1</sup>, P. van Rijn<sup>2</sup>, P. Leijts<sup>3</sup> and M. Goffredo<sup>1</sup>.

<sup>1</sup>Istituto Zooprofilattico Sperimentale “G. Caporale”, Teramo, Italy, <sup>2</sup>Food and Consumer Product Safety, The Netherlands, <sup>3</sup>Wageningen UR Central Institute for Animal Disease Control, The Netherlands

Bluetongue is an orbiviral disease affecting domesticated ruminant livestock, especially sheep and cattle (Erasmus 1990; MacLachlan 1994). The disease occurs almost worldwide (Tabachnick 2004) and is transmitted by some 30 species of biting midges of the genus *Culicoides* (Meiswinkel et. al. 2004). Bluetongue virus (BTV) is represented by 24 serotypes; concerning Europe eight of these occur in the Mediterranean Basin where they are confined to below the 45<sup>th</sup> parallel. Within this region four species of *Culicoides* have been incriminated as vectors: the Afro-Asiatic *C. imicola* (accounting for at least 90% of disease transmission) and the palaeartic endemics *C. obsoletus*, *C. scoticus* and *C. pulicaris* (Mellor and Pitzolis 1979; Caracappa et. al. 2003; Savini et. al. 2005). Some workers (Purse et. al. 2005) suggest that *C. imicola* – under the influence of a warming global climate – may move further northwards into Europe. If true, this poses an increased future risk to livestock production in the region.

Since 1998 five serotypes of BTV have swept across the Mediterranean Basin and affected 15 countries many of which had never experienced the disease previously (Purse et. al. 2005). In August 2006 – following upon an unprecedented warm summer – BT took a significant ‘leap’ northwards appearing unexpectedly in central Europe and affecting parts of the Netherlands, Belgium, Germany and northern France. To date the disease has spread across approximately 100 000 km<sup>2</sup> of territory and advanced almost to the 53<sup>rd</sup> parallel, the furthest north BTV has penetrated anywhere in the world. The virus serotype was identified by the European Community Reference Laboratory (Pirbright, England) as BTV-8 and found to be closely related phylogenetically to a 1982 West African strain of the same serotype; importantly, it appears not descended from vaccine forms of BTV-8 used in parts of southern Europe some years ago (D. Cavanagh, ProMed-mail, archive no. 20060828.2448). The link to sub-Saharan Africa, and the fact that BTV-8 is novel to Europe, indicates the virus was introduced over a long distance. But from exactly where and how is not known.

In September 2006 the veterinary authorities of the Netherlands in conjunction with the Istituto Zooprofilattico Sperimentale (IZS), Teramo, Italy, commenced light trapping (using the Onderstepoort-type blacklight trap) on a BT-affected dairy in Gulpen, south-east Limburg in an attempt to detect the virus in pools of identified *Culicoides*. In 35 collections made between 14-24 September, 12 001 midges (representing 15 species) were captured. The average percentage prevalences and parity rates (as derived from a single randomly chosen light trap collection) of the seven commonest *Culicoides* species captured in Gulpen are listed in **Table 1** (where they are assigned also to subgenus and species complex). Those taxa that could not be identified reliably on external morphology were lumped into mixed species pools; these are *C. obsoletus/C. scoticus* and *C. pulicaris/C. lupicaris* and include species previously incriminated in the transmission of BTV in the Mediterranean Basin (Mellor and Pitzolis 1979; Savini et. al. 2005; De Liberato et. al. 2005). All captured midges were age-graded as either nulliparous, parous, gravid or freshly bloodfed following the method of Dyce (1969); the parity rate is the percentage of older parous and gravid females in the population i.e. the individuals most likely to harbour BTV. After 11 days of continuous light trapping a cumulative total of 3 242 older parous and gravid females

were obtained and subdivided amongst 40 species pools for virus detection (Table 1). The clear majority of pools comprised *C. obsoletus/C. scoticus* (77.1%) and *C. dewulfi* (18.2%). The pools were code numbered and then submitted ‘blind’ to the Central Institute for Animal Disease Control (CIDC), Lelystad on 29 September to be tested individually using an in-house BT serogroup-specific RT-PCR (based on segment 10 of the non-structural protein NS3).

On the 4<sup>th</sup> of October one pool of *C. (Avaritia) dewulfi* was found PCR+ to BTV. This species has never before been linked to the transmission of BT disease in Europe. While *C. dewulfi* remains to be incriminated more conclusively as a novel vector the result is of some interest:

- *C. dewulfi* is known to breed in “...cow dung lying naturally in the field” (Kettle and Lawson 1952). This dependence on bovid dung means that the distribution of *C. dewulfi* will be determined by that of cattle. This implies that *C. dewulfi* will be found throughout most of the Netherlands – and the rest of Europe - where cattle are husbanded;
- This cattle-*Culicoides* association is paralleled in other parts of the world affected by BT such as south-east Asia (including Australia) and Africa where the respective vectors *C. brevitarsis* and *C. bolitinos* breed also in cattle and buffalo dung (Meiswinkel 1989). It is germane to point out that *C. bolitinos* is also an important vector of the devastating disease of African horse sickness (AHS) (Meiswinkel and Paweska 2003) because, as demonstrated in Ireland, *C. dewulfi* feeds avidly also on the horse (Townley et. al. 1984) and breeds in its dung (Kettle 1962);
- *C. dewulfi* was described in 1936 from material collected in Belgium but – remarkably - has never before been reported from the Netherlands. It occurs principally in temperate latitude countries being less commonly reported further south along the northern margin of the Mediterranean Basin. Throughout the region very little is known about the local abundance levels and seasonal dynamics of *C. dewulfi*;
- As a taxonomic entity *C. dewulfi* belongs within the subgenus *Avaritia*, which, across the world, is subdivided into at least 10 species complexes (Meiswinkel et. al. 2004). However, most authors erroneously place *C. dewulfi* within the *Obsoletus* Complex (or the *C. obsoletus* group). Recent morphological and molecular studies (Gomulski et. al. 2005) conducted in Italy clarified further its position within the Palaearctic sector of *Avaritia* and showed it to be: 1. a monophyletic taxon within *Avaritia*, and 2. only distantly related to the six species that comprise the *Obsoletus* Complex *sensu stricto*;
- Similarly, as an entity in the field, most researchers in virus isolation or vector competency studies are not able to confidently distinguish *C. dewulfi* from other sympatrically captured species of the subgenus *Avaritia* including *C. obsoletus* and *C. chiopterus*. This failure might explain the significant BTV oral susceptibility differences measured recently amongst United Kingdom populations of *C. obsoletus* (Carpenter et. al. 2006). Accurate identification - i.e. a sound taxonomy - is fundamental to such studies and exposes the lack of taxonomic expertise currently available in Europe (as was so clearly articulated by the Vector Working Group during the Third International Symposium on Bluetongue in Taormina, Sicily, 2003);
- The incrimination of *C. dewulfi* as a vector of BTV does not exclude other species, or species complexes, from having played a role in the outbreak in central Europe. Judging from its high parity rate (38.6%) it would be prudent to assume that the *Obsoletus* Complex was involved also;
- Finally, not a single specimen of the Afro-asiatic *C. imicola* was found at Gulpen nor amongst >29 000 *Culicoides* captured at 104 sites sampled throughout the Netherlands. Although *C. imicola* appears not to have established itself in central Europe this does not

exclude the possibility of BTV-8 having been brought into the region - on an aeroplane or otherwise - by a single infected individual of *C. imicola*.

Although additional studies are required to more conclusively implicate *C. dewulfi* as a vector of BTV it now seems more clear that *Culicoides* endemic to central Europe are quite capable of replicating and transmitting the virus and that they can do so without the ‘help’ of *C. imicola*. Furthermore, because the vectorial capacity of *Culicoides* will likely increase under the influence of a globally warming climate, it is fit to observe that the remaining most-abundant species of *Culicoides* captured at Gulpen have already been implicated in the transmission of BTV elsewhere in Europe.

Central Europe must therefore consider itself vulnerable to a variety of *Culicoides*-borne orbiviral diseases of livestock and which may be introduced adventitiously along any one of its numerous trade routes. This includes the truly devastating disease of African horse sickness. As mentioned above *C. dewulfi* feeds avidly also on horses.

**Table 1. *Culicoides* species (including subgenus and species complex designation) and the number and size of pools tested for bluetongue virus (BTV) using a serogroup-specific PCR; Gulpen, Limbourg province, s.e. Netherlands, September/October, 2006 (na = not applicable).**

<i>Culicoides</i> species	Total parous/gravid specimens	Total pools	Pool size	% light trap prevalence	% parous/gravid (parity rate)	<i>Culicoides</i> subgenus	Species complex	PCR (+/-)
<i>obsoletus/scoticus</i>	2 500	25	100	68.5	38.6	<i>Avaritia</i>	Obsoletus	-
<i>dewulfi</i>	590	12	40/50	17.4	42.0	<i>Avaritia</i>	Dewulfi	+
<i>chiopterus</i>	36	1	36	0.4	50.0	<i>Avaritia</i>	Chiopterus	-
<i>pulicaris/lupicaris</i>	96	1	96	12.5	11.1	<i>Culicoides</i>	Pulicaris	-
<i>punctatus</i>	20	1	20	0.4	25.0	<i>Culicoides</i>	Pulicaris	-
<i>imicola</i>	0	0	0	0	0	<i>Avaritia</i>	Imicola	na

## References

- Carpenter S, Lunt HL, Arav D, Venter GJ. and Mellor PS 2006 Oral susceptibility to bluetongue virus of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *Medical and Veterinary Entomology* 43, 73-78
- Caracappa S, Torina A, Guercio A, Vitale F, Calabrò A, Purpari G, Ferrantelli V, Vitale M and Mellor, PS 2003. Identification of a novel bluetongue virus vector species of *Culicoides*. *Veterinary Record* 153: 71-74
- De Liberato C, Scavia G, Lorenzetti R, Scaramozzino P, Amaddeo D, Cardati G, Scicluna M, Ferrari G and Autorino GL 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *Veterinary Record* 156: 301-304
- Dyce, AL 1969. Recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *Journal of the Australian Entomological Society* 8: 11-15
- Erasmus, BJ 1990 Bluetongue virus. In *Virus Infections of Ruminants* vol. 3. Eds Z. Dinter and B. Morein. Elsevier Science Publishers, New York. pp. 227-237
- Gomulski LM, Meiswinkel R, Delécolle J-C, Goffredo M and Gasperi G 2005. Phylogenetic relationships of the subgenus *Avaritia* Fox, 1955 including *Culicoides obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2 ribosomal DNA sequences. *Systematic Entomology* 10: 619-631
- Kettle DS 1962. The bionomics and control of *Culicoides* and *Leptoconops* (Diptera, Ceratopogonidae = Heleidae). *Annual Review of Entomology* 7: 401-418
- Kettle DS and Lawson JWH 1952. The early stages of British biting midges *Culicoides* Latreille (Diptera: Ceratopogonidae) and allied genera. *Bulletin of Entomological Research* 43: 421-467
- Maclachlan N J 1994. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comparative Immunology of Microbial Infectious Diseases* 17: 197-206
- Meiswinkel R. 1989. Afrotropical *Culicoides*: a redescription of *C. (Avaritia) imicola* Kieffer, 1913 (Diptera: Ceratopogonidae) with description of the closely allied *C. (A.) bolitinos* sp. nov. reared from the dung of the African buffalo, blue wildebeest and cattle in South Africa. *Onderstepoort Journal of Veterinary Research* 56: 23-39
- Meiswinkel R, Gomulski LM, Delécolle J-C, Goffredo M and Gasperi G 2004. The taxonomy of *Culicoides* vector complexes – unfinished business. In *Bluetongue, Part I*. Eds N. J. MacLachlan and J. E. Pearson. *Proceedings Third International Symposium, Taormina, 26-29 October 2003*. *Veterinaria Italiana* 40: 151-159
- Meiswinkel R and Paweska J 2003. Evidence for a new field *Culicoides* vector of African horse sickness in South Africa. *Preventive Veterinary Medicine* 60: 243-253

Mellor PS and Pitzolis G 1979. Observations on breeding sites and light-trap collections of *Culicoides* during an outbreak of bluetongue in Cyprus. *Bulletin of Entomological Research* 69: 229-234

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PC and Baylis M 2005. Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews, Microbiology* 3: 171-181

Savini G, Goffredo M, Monaco F, Di Gennaro A, Cafiero MA, Baldi L, De Santis P, Meiswinkel R and Caporale V 2005. Bluetongue virus isolations from midges belonging to the *Obsoletus* complex (*Culicoides*, Diptera: Ceratopogonidae) in Italy. *Veterinary Record* 157: 133-143

Tabachnick WJ 2004. *Culicoides* and the global epidemiology of bluetongue virus infection. *Veterinaria Italiana* 40. 145-150

Townley P, Baker, KP and Quinn PJ 1984. Preferential landing and engorging sites of *Culicoides* species landing on a horse in Ireland. *Equine Veterinary Journal* 16: 117-120

## **Annex C to Appendix 9**

### **Entomological monitoring of *Culicoides* species in Belgium and the Grand Duchy of Luxembourg**

*M. Madder*<sup>1</sup>, *R. De Deken*<sup>1</sup>, *G. De Deken*<sup>1</sup>, *C. Fassotte*<sup>2</sup>, *R. Cors*<sup>2</sup>, *T. Defrance*<sup>2</sup>,  
*M. Cavelier*<sup>2</sup>, *B. Losson*<sup>3</sup>, *E. Haubruge*<sup>4</sup>, *J. Bortels*<sup>4</sup>, *F. Francis*<sup>4</sup>, *A. Besch*<sup>5</sup>

<sup>1</sup>ITMA, Belgium; <sup>2</sup>Wallon Agricultural Research Center, Gembloux, Belgium;  
<sup>3</sup>University of Liège, Liège, <sup>4</sup>Gembloux Agricultural University, Gembloux,  
Belgium; <sup>5</sup>Ministère de l'agriculture, de la viticulture et du développement  
rural, Luxembourg;

## Introduction and Objectives

Since the recent outbreaks of Bluetongue (BT) in Western Europe in August 2006, many efforts have been made in Belgium, The Netherlands, Germany and Luxembourg to identify and monitor possible vectors of the disease, in particular biting midges of the genus *Culicoides*.

This report deals with the sampling techniques used in Belgium and the Grand Duchy of Luxembourg, the results of the sampling carried out in 2006, the identification of the *Culicoides* spp. captured and some considerations about possible vectors in the outbreak area.

Several institutes and universities were involved in this study:

- The Department Animal Health of ITMA for the cross-sectional study using mobile traps deployed near bluetongue infected herds.
- The Department Biological Control and Plant Genetic Resources of the Walloon Agricultural Research Center (Ir C. Fassotte) for the trappings carried out with two large immobile Rothamsted insect survey suction traps.
- The Department of Parasitology of the University of Liege and the Department of functional and evolutionary Entomology (Gembloux Agricultural University) for a longitudinal study in two different habitats.
- Dr Arthur Besch of the « Ministère de l'agriculture, de la viticulture et du développement rural au Luxembourg » organised sampling in Luxembourg.

## Materials and Methods

### Trapping of *Culicoides*

Several vector collections were brought into play to monitor the distribution (cross-sectional survey) and phenology (longitudinal survey) of *Culicoides* species in Belgium and the Grand Duchy of Luxembourg.

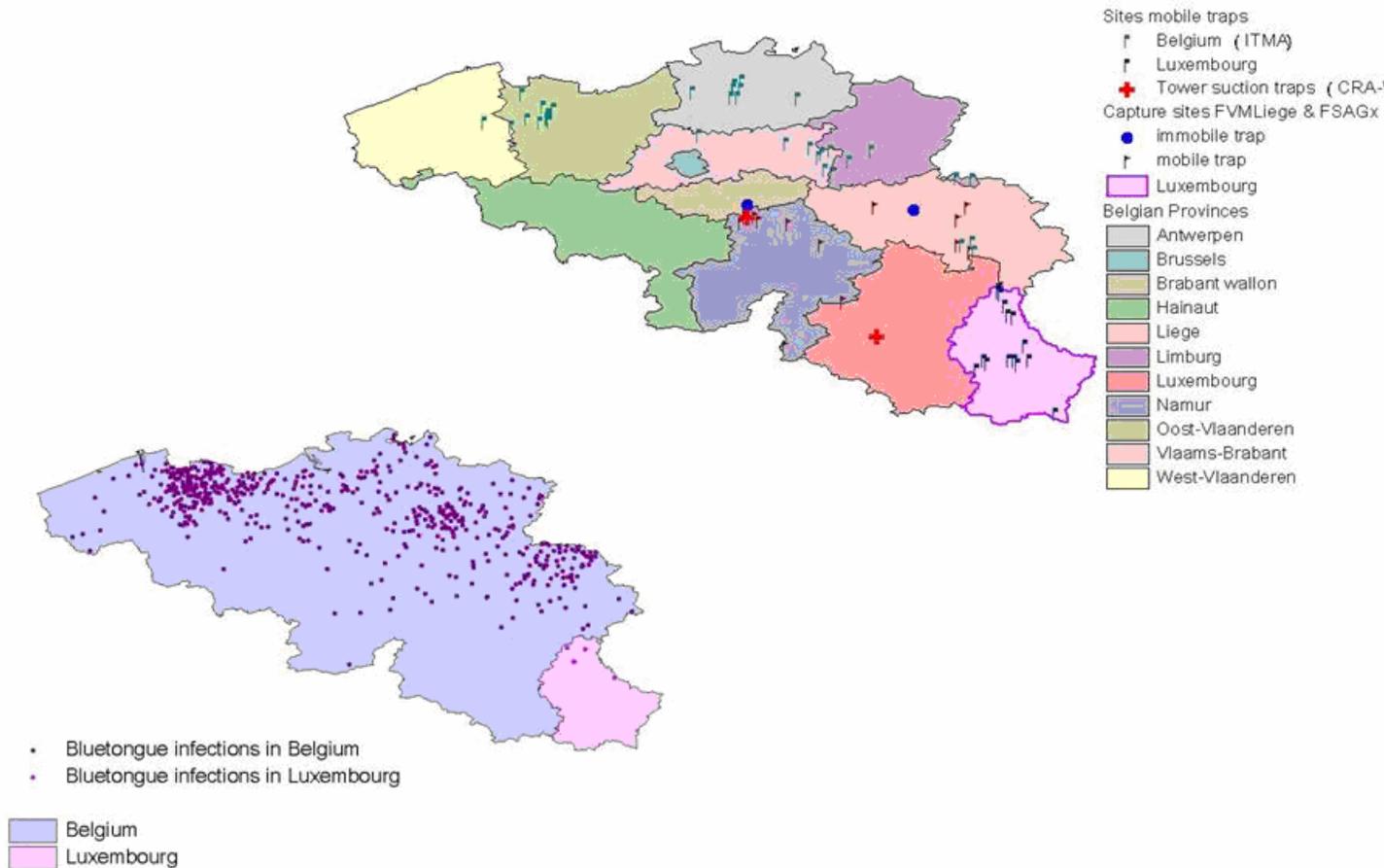
### Trapping with the mobile “John Hopkins – model 1212” traps

Short after the first cases of BT were confirmed in August 2006 a cross-sectional study was started with five “John Hopkins” traps deployed near recent bluetongue outbreaks to identify possible vectors. These traps are equipped with a ultraviolet light tube attracting insects and a fan to push them down in a sample bottle filled with 70% alcohol. The light was switched on automatically at dusk and switched off at dawn by a light sensor. No insects were caught during daytime with this type of trap.

The main objective for this study was to catch and identify different *Culicoides* species near herds, where infected animals had been identified, and to determine their possible role in the transmission of Bluetongue virus. The collections were identified up to species level. Initially, the collections were performed in the province of Liege and Limburg (**Figure 1**) where the first outbreaks occurred. The traps were always deployed close to the infected animals and emptied every two days. Generally, every week traps were moved to a new outbreak area.



During the period of August to December the sampling in Belgium followed the spread of the disease westwards. A comparison was made between the distribution of *Culicoides* determined by ITMA in 2006 and the observations of Goethgebuer in 1951. This study was coordinated and performed by Department Animal Health of ITMA.



**Figure 1. Distribution traps and bluetongue cases 2006**

### Trapping with “south-African - OVI” traps

Two traps of the OVI (Onderstepoort Veterinary Institute, South Africa) type were used in Wallonia, the southern part of Belgium, from the 11<sup>th</sup> of September 2006 until the end of the year. The objective was to monitor the phenology of possible vectors of BT in a longitudinal study with immobile traps irrespective of the presence of BT outbreaks in Wallonia. A longitudinal study (from September to November) was performed at two sites, Sart Tilman and Walhain, while from time to time some occasional sampling occurred in other sites of the provinces of Liege and Brabant Wallon (**Figure 1**). In addition, during the months of November, December 2006 and January 2007 sampling was carried out indoors in closed and open stables.

Insects sampled in Wallonia were collected in a beaker containing water and a few drops of detergent as a wetting agent. In the morning catches were collected and decanted into a cup containing 80% ethanol. The traps were set as close as possible to host animals at a height of

between 1.5 m to 2.50 m above the ground and outdoors. Once catches became very rare outdoors (in December), sampling was pursued inside animal housings. Currently the specimens captured with these traps were only identified up to genus level.



The south-African traps differ from the “John Hopkins” traps in several ways. Most importantly the efficacy of the south-African traps is much higher due to the use of a more powerful ultraviolet light tube (double amount of Watts). Traps are powered by a 12 V car battery. Secondly, this type of trap

is not equipped with a light sensor to activate and de-activate the trap. As a consequence, the traps were activated manually one hour before dusk and switched off and emptied around 9.00 a.m. The better efficacy and longer operation period of the south-African trap were probably responsible for the much larger numbers of *Culicoides* captured by these traps.

The Faculty of Veterinary Medicine of the University of Liege and the Gembloux Agricultural University organised this part of the study.

#### Trapping with immobile suction traps

In Belgium, two immobile Rothamsted insect survey suction traps or “tower suction traps” are in use to monitor daily wind-borne insects at a height of 12 m. They are located in Gembloux and Libramont (**Figure 1**).

Since the 1<sup>st</sup> of May 2006 and until the 25 March 2007 samples were collected in Gembloux and screened for *Culicoides*. In Libramont only samples from 12 September until 15 October 2006 and from 13 December 2006 until 11 March 2007 were available for identification. Currently all these samples were identified up to species level. The number and sex of the *Culicoides* was determined as well as their physiological status (nutritional stage and presence or absence of eggs).

The main purpose of these trapping is to search for correlations between the dispersal of *Culicoides* species and ecological or physiological parameters. Another aim is to study the suitability of the suction trap as a rapid alert system for the survey of *Culicoides* emergence.

The Walloon Agricultural Research Center was responsible for the collection with the immobile suction traps.

#### Trapping with “artisanal” traps

In the Grand Duchy of Luxembourg artisanal traps have been used to monitor the *Culicoides* activity between the 1<sup>st</sup> and 12<sup>th</sup> of September 2006. The traps were equipped with an ultraviolet light tube but no detailed description of the traps was available. Traps were activated around sunset until the following morning.

Since no outbreaks were recorded during the sampling period, the only aim of the sampling was to get an idea about the *Culicoides spp.* present in the region.

Sampling was organized by the Ministry of Agriculture of the Grand Duchy of Luxembourg and the National Museum of Natural History of Munster.

#### Species identification

To improve the identification of captured insects and the knowledge on *Culicoides* a short course (2 days) in *Culicoides* biology and identification was organised by and at ITM for entomologists from Belgium and surrounding countries. Jean Claude Delécolle (Musée de Zoologie de Strasbourg, France), one of the authorities in the field of *Culicoides*, was invited to lecture the course.

All collected samples were stored in 70% alcohol and examined under a stereomicroscope. For identification, members of the genus *Culicoides* were first separated from other insect genera in each sample and then further identified up to species or species complexes' level. In some samples the feeding status of the midge, its sex and the occurrence of eggs in female specimens was recorded.



A training day of mounting *Culicoides* specimens (mainly females) for identification up to species level was organised at CRA-W in Gembloux for all technicians involved in the monitoring group (after a former training of one technician by Jean-Claude Delécolle).

## Results

### Trapping with the mobile “John Hopkins” traps

During the entire sampling period from 26 August to 31 December a total number of 1959 *Culicoides* were caught of which 1538 and 421 were female and male individuals, respectively. In total 16 different species were identified in Belgium of which the most important species belonged to the *C. obsoletus-complex s.l.*; *C. obsoletus*, *C. dewulfi*, *C. chiopterus* and *C. scoticus* or to the following species: *C. pulicaris*, *C. festivipennis* and *C. punctatus*.

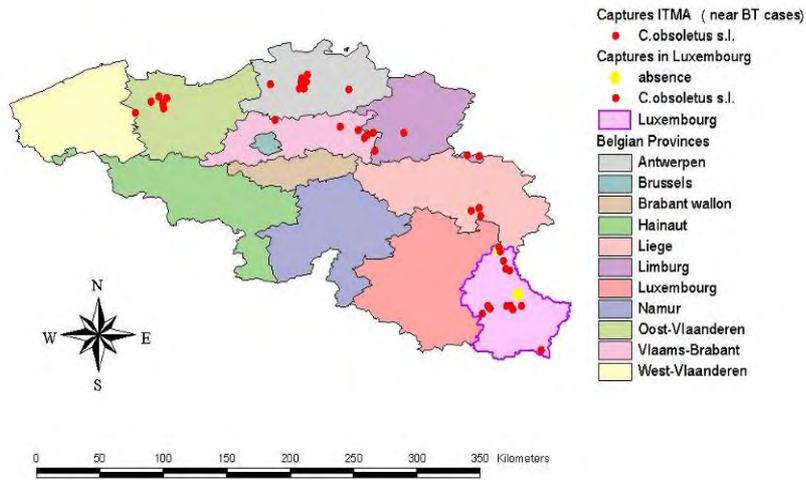
The number of *Culicoides* caught per species is presented in the **Table 1** below:

**Table 1. Number of different *Culicoides* species caught in Belgium in 2006**

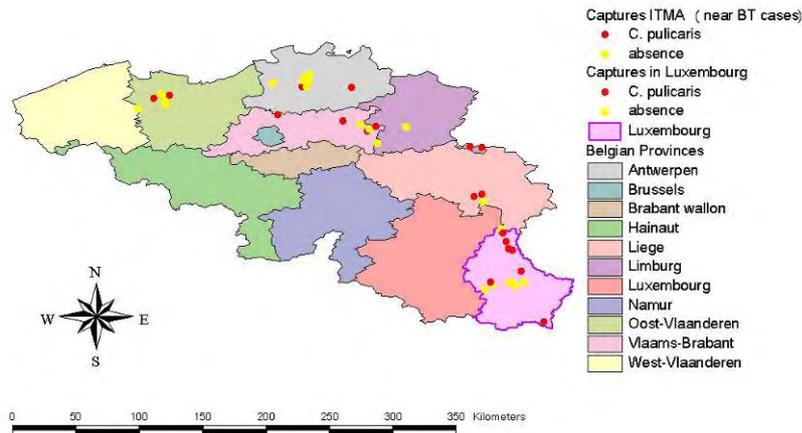
<i>C obsoletus complex s.l.</i>	1499
<i>C scoticus (males)</i>	25
<i>C dewulfi (males)</i>	71
<i>C chiopterus</i>	46
<i>C pulicaris</i>	111
<i>C festivipennis</i>	109
<i>C punctatus</i>	75
<i>C nubeculosis</i>	11
<i>C pallidicornis</i>	3
<i>C riethi</i>	2
<i>C stigma</i>	2
<i>C lupicaris</i>	1
<i>C parroti</i>	1
<i>C circumscriptus</i>	1
<i>C fascipennis</i>	1
<i>C salinarius</i>	1

The distribution of the *Culicoides* species captured by ITMA from August to December 2006 in Belgium and those captured in Luxembourg is presented in **Figure 2**.

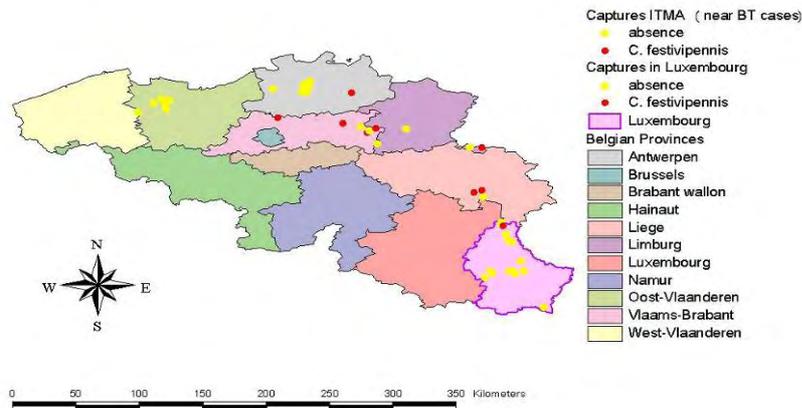
## Distribution *C. obsoletus* s.l. 2006



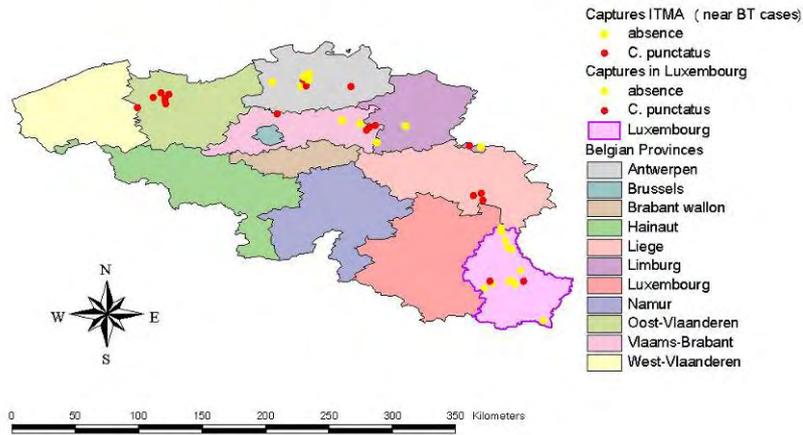
## Distribution *C. pulicaris* 2006



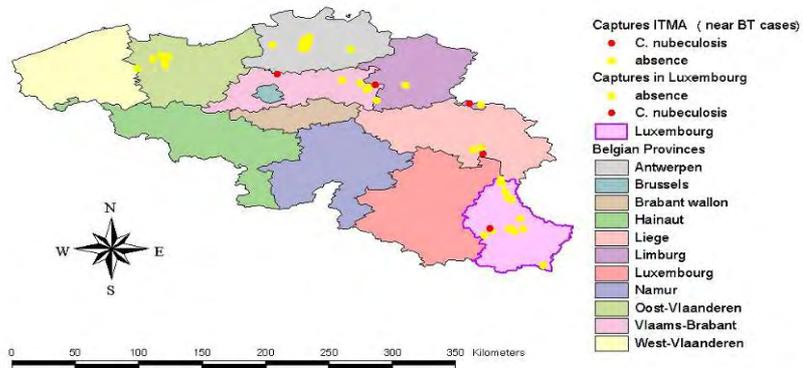
## Distribution *C. festipennis* 2006



## Distribution *C. punctatus* 2006



## Distribution *C. nubeculosis* 2006



## Distribution rare *Culicoides* spp. 2006

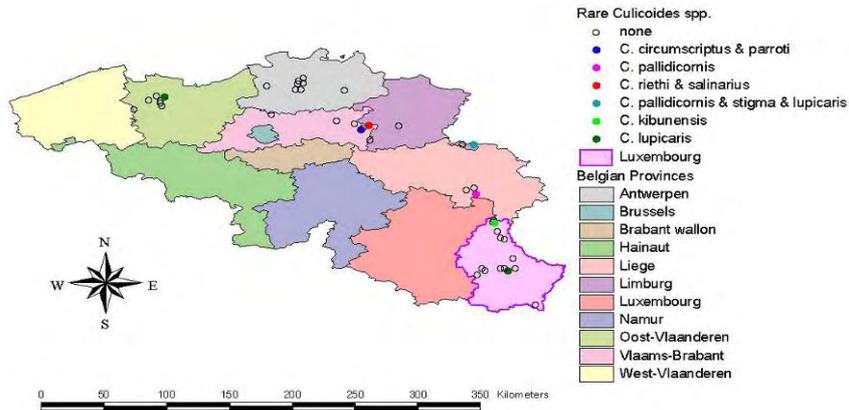


Figure .distribution traps and bluetongue cases 2006

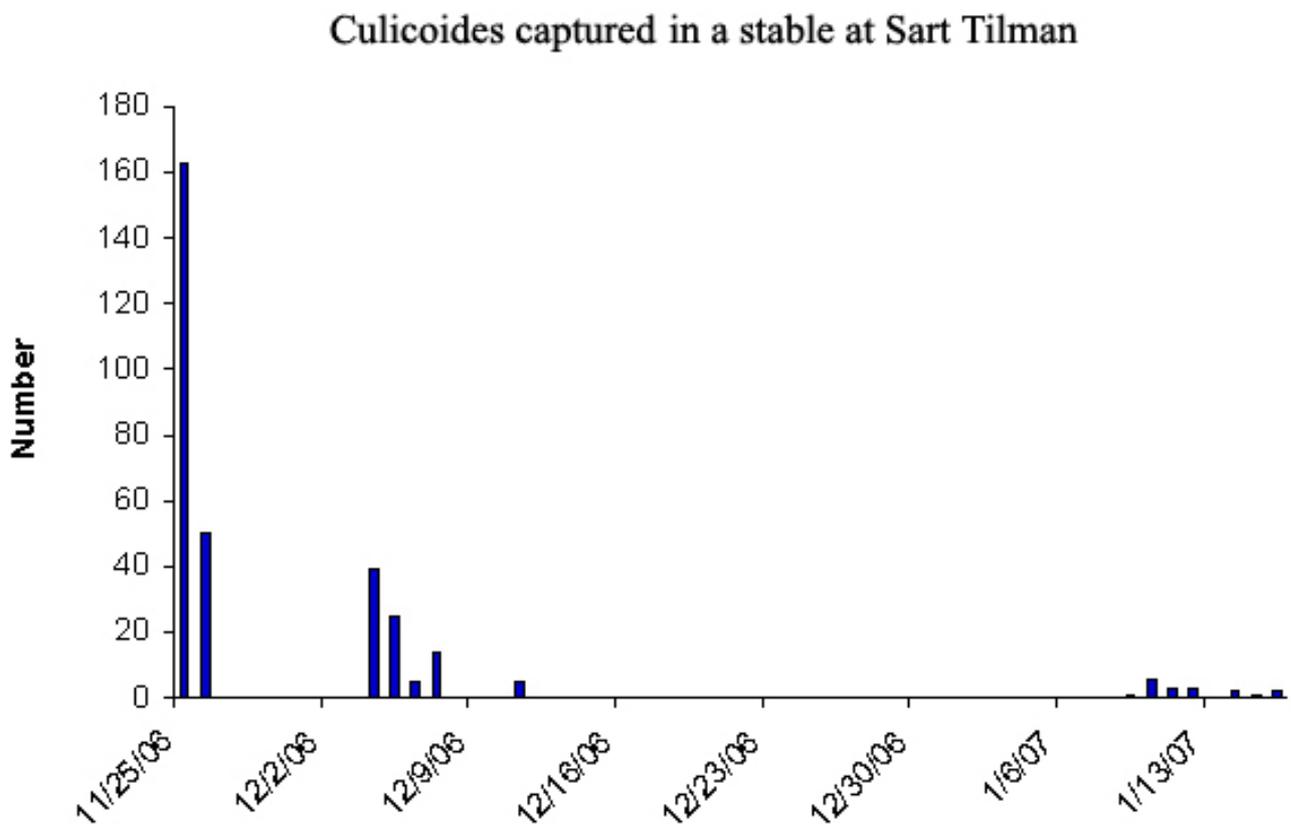
Trapping with “south-African - OVI” traps

*A: Trapping indoors:*

During a longitudinal study *Culicoides* were sampled 5 times and 14 times respectively in an open stable at Gembloux and in a closed stable at Sart Tilman.

In the open stable at Gembloux, 113 *Culicoides* were sampled between 24 November and 20 December 2006. Most of these *Culicoides* were caught during 2 nights of trapping in November (99%). After December 20<sup>th</sup> no more *Culicoides* were trapped in this stable.

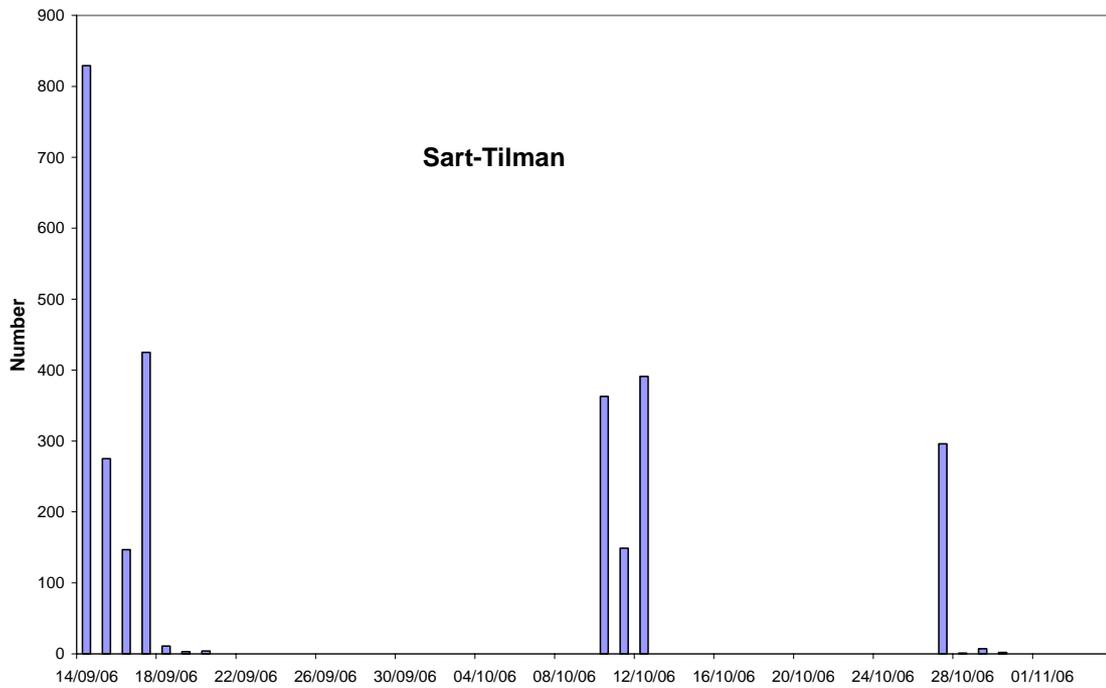
The study at Sart Tilman started the 25<sup>th</sup> of November 2006 and ended the 16<sup>th</sup> of January 2007. Trapping resulted in 319 captured specimens. Most of these *Culicoides* were caught during 2 nights of trapping in November (66%), in December around 28% of them was captured during a period of 6 nights and even in January 2007 a total of 18 (6%) *Culicoides* were found in the traps during a period of one week (**Figure 3**). No identification up to species level was done yet for this collection.



**Figure 3. Number of *Culicoides* caught in OVI traps in closed stable at Sart Tilman.**

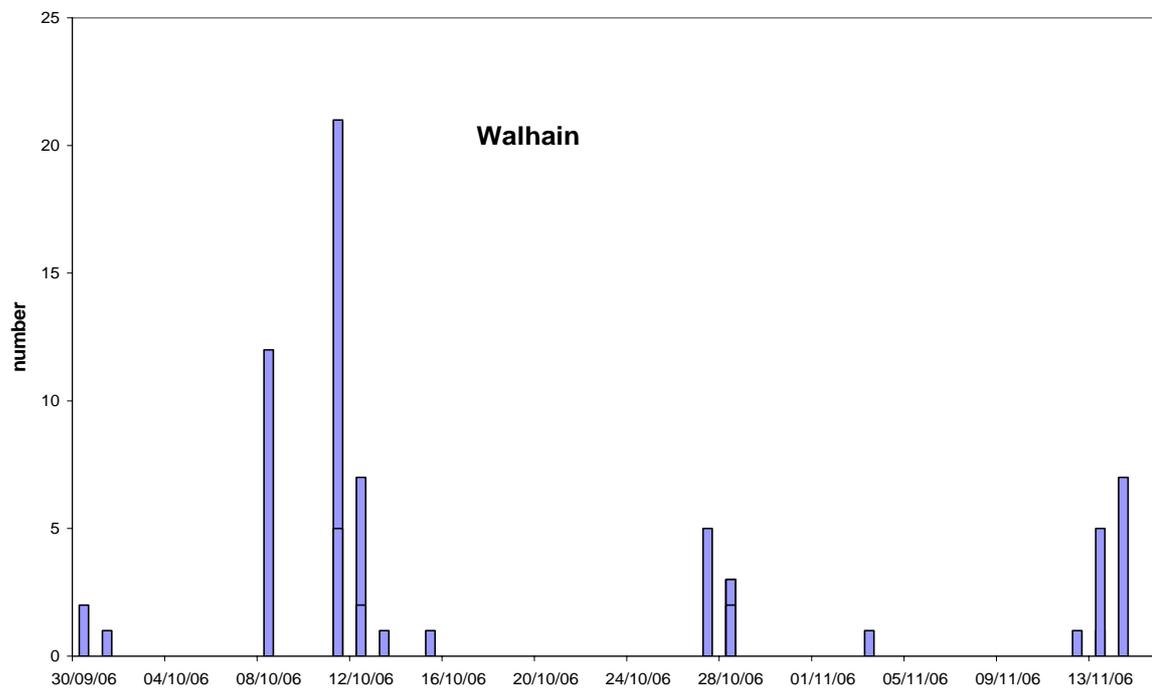
*B. Trapping outdoors:*

In a second longitudinal study at Sart Tilman the OVI trap was placed outside near a river where cattle were grazing. The study started the 14<sup>th</sup> of September and ended in November when the traps could not capture any midge. The number of *Culicoides* caught is presented in **Figure 4**. Most of the *Culicoides* were captured in September with a maximum of 829 individuals caught in one night. The numbers of midges captured decreased rapidly at the end of October when temperatures at night fell suddenly under 5°C. From November onwards no midges could be trapped outdoors at Sart Tilman. Thereafter the traps were transferred into the stables where the capture of midges resumed.



**Figure 4. Number of *Culicoides* caught outdoors in OVI traps at Sart Tilman.**

Another longitudinal study was carried out in Walhain near a meadow with horses and sheep. The results are presented in **Figure 5**. The overall number of *Culicoides* caught in this area was significantly lower compared to Sart Tilman. The highest number of individuals trapped in a single night was 21. The last *Culicoides* was found on the 14<sup>th</sup> of November.



**Figure 5. Number of *Culicoides* caught outdoors in OVI traps at Walhain**

During the longitudinal studies in the provinces of Liege and Walloon Brabant, 942 individuals (77% females and 23% males) belonging to 11 different *Culicoides* species were identified. More than 76% of the captured individuals belonged to the *C. obsoletus-complex*. Members of the following species were also observed: *C. chiopterus*, *C. dewulfi*, *C. festivipennis*, *C. punctatus* or *C. pulicaris*.

Looking at the cross-sectional study in Wallonia, an overall trend of decreasing number of *Culicoides* towards the end of the year is observed. However a remarkable peak in the numbers of captured midges is observed around the 10<sup>th</sup> of October. This coincides with a period of warm weather, when the maximum temperatures during daytime exceeded 20°C and the minimum temperature at night fluctuated around 14°C. From half November onwards, the trap was moved indoors towards several types of open or closed stables. The last *Culicoides* caught outdoors in Wallonia was captured on the 20<sup>th</sup> of December.

*Trapping with immobile suction traps*

The data of the immobile suction traps are presented in **Table 2** and **3**.

**Table 2. Number of *Culicoides* caught with Rothamsted suction traps in 2006.**

Month of sampling	males	females	Total
<b>Trap at Gembloux</b>	<b>Total: 159 (23%)</b>	<b>Total: 532 (77%)</b>	<b>Total: 691</b>
May 2006	21	33	54
June 2006	69	224	293
July 2006	9	102	111
August 2006	8	43	51
September 2006	31	81	112
October 2006	15	33	48
November 2006	4	11	15
December 2006	0	1	1
<b>January 2007</b>	<b>0</b>	<b>2</b>	<b>2</b>
<b>February 2007</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>March 2007</b>	<b>2</b>	<b>2</b>	<b>4</b>
<b>Trap at Libramont</b>	<b>Total: 24 (25%)</b>	<b>Total: 73 (75%)</b>	<b>Total: 97</b>
September 2006	18	58	76
October 2006	6	15	21
November 2006		no data	
December 2006	0	0	0
January 2007	0	0	0
February 2007	0	0	0
March 2007	0	0	0

**Table 3. Percentage of *Culicoides* that never had a bloodmeal (nulliparous), those that have blood in their abdomen, those that have eggs and blood and those that only have eggs in their abdomen.**

Month of Sampling Gembloux	nulliparous	blood	eggs/blood	eggs
May 2006	33.3%	45.5%	21.2%	0.0%
June 2006	45.1%	25.9%	24.6%	1.3%
July 2006	26.5%	21.6%	42.2%	3.9%
August 2006	53.5%	14.0%	2.3%	11.6%
September 2006	56.8%	27.2%	8.6%	0.0%
October 2006	45.5%	42.4%	9.1%	3.0%
November 2006	63.6%	36.4%	0.0%	0.0%
December 2006	0.0%	100.0%	0.0%	0.0%
<b>January 2007</b>	<b>50.0%</b>	<b>0.0%</b>	<b>50.0%</b>	<b>0.0%</b>
<b>February 2007</b>	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>
<b>March 2007</b>	<b>100.0%</b>	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>
<b>Libramont</b>				
September 2006	56.9%	24.1%	15.5%	3.4%
October 2006	37.5%	25.0%	37.5%	0.0%
November 2006	no data			
December 2006	0.0%	0.0%	0.0%	0.0%
January 2007	0.0%	0.0%	0.0%	0.0%
February 2007	0.0%	0.0%	0.0%	0.0%
March 2007	0.0%	0.0%	0.0%	0.0%

The sex ratio for the *Culicoides* caught by this type of trap is 1:3.3 (males:females). The overall number of individuals caught was highest in June with about 300 *Culicoides* per month and thereafter decreased gradually until January when only 2 midges were captured. During the month of August the number of *Culicoides* was lower than expected. In February 2007 no midges were captured. Then 3 midges were captured on 1, 11 and 13 March 2007.

The following species have been identified in captures at Gembloux and Libramont (788 specimens examined) (**Table 4**). Up to now seven species (marked with \*) were caught with the suction trap only.

**Table 4. *Culicoides* spp. captured with the suction trap at Gembloux and Libramont in 2006-2007.**

<i>C. obsoletus</i> complex s.l.(= <i>obsoletus</i> , <i>scoticus</i> , <i>chiopterus</i> et <i>dewulfi</i> )	7
<i>C. obsoletus</i>	474
<i>C. scoticus</i>	
<i>C. chiopterus</i>	96
<i>C. dewulfi</i>	41
<i>C. punctatus</i>	65
<i>C. stigma</i>	18
<i>C. pulicaris</i>	16
<i>C. kibunensis</i> *	13
<i>C. minutissimus</i> *	4
<i>C. circumscriptus</i>	4
<i>C. lupicaris</i>	5
<i>C. subfascipennis</i> * / <i>pallidicornis</i>	4
<i>C. deltus</i> *	3
<i>C. festivipennis</i>	3
<i>C. achrayi</i> *	2
<i>C. newsteadi</i> *	1
<i>C. salinarius</i>	1
<i>C. vexans</i> *	1

The proportion of *Culicoides* that never had a blood meal was around 45%. About 30% presented traces of blood in their abdomen, 22% both showed blood and eggs and around 3% only had eggs.

#### Trapping with artisanal traps in Luxembourg

Between the 1<sup>st</sup> and the 12<sup>th</sup> of September 2006, 81 *Culicoides* were caught in the traps used in the Grand Duchy of Luxembourg. 81% of these midges were females and 19% were males. The identification up to species level revealed the following numbers per species:

**Table 5 *Culicoides* spp. captured in September 2006 in Luxembourg**

<i>C. obsoletus</i> complex s.l.	54
<i>C. dewulfi</i> (males)	2
<i>C. pulicaris</i>	17
<i>C. nubeculosis</i>	3
<i>C. punctatus</i>	2
<i>C. festivipennis</i>	1
<i>C. lupicaris</i>	1
<i>C. kibunensis</i>	1

The distribution of the different species over the different sampling sites is presented in **Figure 2**.

#### Comparison of the actual and historical distribution of *Culicoides*

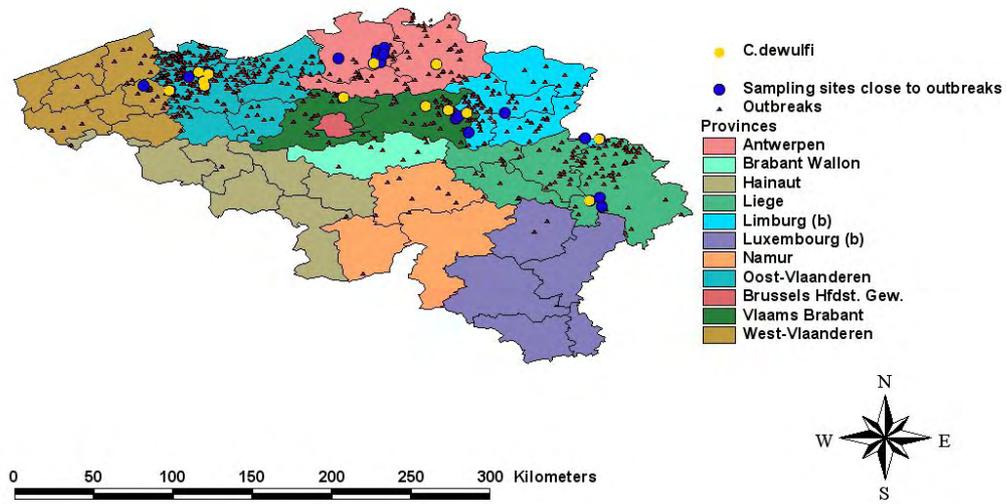
The actual distribution of *Culicoides* spp., as stated by the results of the sampling carried out by ITMA in 2006 near 28 bluetongue infected herds, was compared to a former study performed by Goethgebuer in 1950 (Goetghebuer, 1952, Le genre *Culicoides* - diptères *Ceratopogonides* - et ses

représentants en Belgique, Biologisch jaarboek – Dodonaea, 19, 185-191). This author sampled *Culicoides spp.* at 31 different sites scattered over Belgium.

Comparison of the actual and former distribution of the different species revealed two striking differences. The first one concerns the actual and former distribution of *C.dewulfi* which was recently identified as the vector (or one of the vectors) of bluetongue in the Netherlands (ProMED-mail post Archive Number: 20061024.3042 - Published on 24-OCT-2006). The distributions can be compared in **Figure 6**.

The second difference was the presence of *C.fascipennis* in 8 of the 31 places visited by Goethgebuer in the 1950's (**Figure 7**), whereas this species was identified only once at one of the sites sampled in 2006.

# Culicoides dewulfi 2006



# Distribution C. dewulfi in 1950's

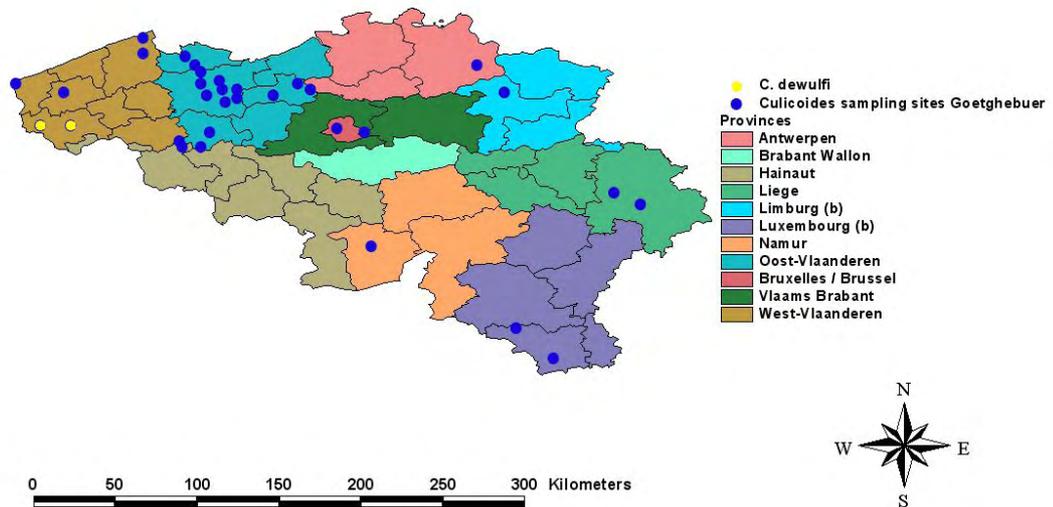


Figure 6. Distribution of C. dewulfi in 2006 and in the 1950's

## Distribution *C. fascipennis* in 1950's

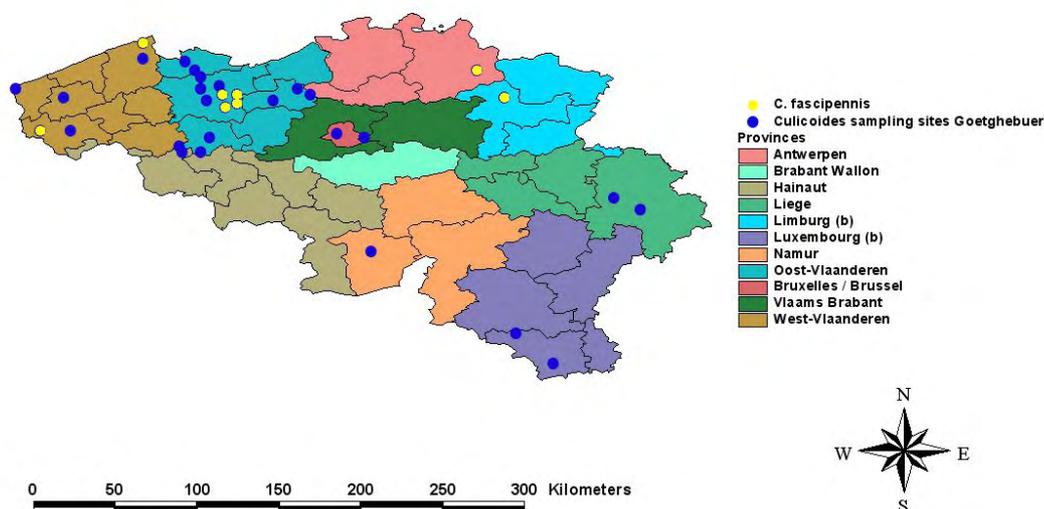


Figure 7. Distribution of the *C. fascipennis* in 1950's in Belgium.

### Discussion and Conclusions

The most abundant *Culicoides* species observed at the outbreak sites during this survey were species belonging to the *C. obsoletus*-complex *s.l.* (*C. obsoletus*, *C. dewulfi*, *C. chiopterus* and *C. scoticus*) and also *C. pulicaris*, *C. festivipennis* and *C. punctatus*.

At all the outbreak sites visited in 2006, where biting midges could be sampled, members of the *C. obsoletus* complex group could be captured, indicating that species within this complex are probably (but therefore not exclusively) vectors of bluetongue. Since the members of this group (especially the females) are extremely difficult to identify up to subspecies level, no conclusive causative link could be identified between a specific species and the bluetongue cases in Belgium.

The study performed with the OVI traps showed that about 75% of the *Culicoides* caught by the traps are females. This is not surprising, as the traps are always deployed close to host animals and only females need a blood meal for reproduction.

Very interesting are the data on *Culicoides* caught in stables. Towards November 2006 the number of *Culicoides* captured by the field traps decreased significantly. The hypothesis was formulated that *Culicoides* to overcome the harsh conditions of winter, would invade stables. This hypothesis is corroborated by the trapping results in stables. Although sampling results became negative outdoors, *Culicoides* were still captured indoors in December and even January. However, the very mild winter of 2006 may have altered the survival chances of biting midges. The prolonged survival of *Culicoides* spp. (essentially species belonging to the *C. obsoletus* complex) is extremely important for a possible recrudescence of the disease in 2007 and the promulgation of regulations to counter disease transmission. The most important questions that arise are: "What will happen to these *Culicoides* and the infection they may harbour". "Can Bluetongue virus survive in these

vectors or does it need to survive in the host population?” and “Are some *Culicoides* species able to reproduce in stables and build up a significant population before spring?”

The data of the Rothamsted suction traps may also reveal interesting information. Since these traps operate at a height of 12 meters, it is hoped that the individuals captured by these traps, will give us an idea about the *Culicoides* that are “ready” to be dispersed by the wind. Therefore, a distinction was made between the different physiological stages of the midges captured by this “tower suction trap”. Only *Culicoides* with a digested blood meal will be able to transmit the BT virus. Results on the species captured by these traps show that those are quite similar to the ones observed in light traps and therefore comparison of capture results obtained by both techniques seems possible.

The limited data on the occurrence of the different *Culicoides* species in the Grand Duchy of Luxembourg showed that a sex ratio of (1:5 males:females) was present in this country. The species identified at 15 sites scattered over Luxembourg were very similar to what was found during the survey in Belgium. In Luxembourg also, the *C. obsoletus* complex was the most abundant group. *Culicoides kibunensis* was the only species identified in Luxembourg, which had not been observed during the 2006 survey in Belgium.

The actual distribution of *Culicoides spp.*, as stated by the survey carried out by ITMA in 2006, was compared to a former study on the distribution of *Culicoides* performed by Goethgebuer in the 1950’s. Notwithstanding this author must have been very acquainted with the species *C. dewulfi*, since he was the first to describe it, he identified this species during his survey in 1950 solely in two villages of the province of West-Vlaanderen. In contrast the distribution of *C. dewulfi* nowadays is either much more common (**Figure 6**) or the link between the presence of *C. dewulfi* and the risk for a site to become an outbreak site is very high. This must be examined more closely.

Surprisingly also: the survey in 2006 detected only one single site where *C. fascipennis* was present in Belgium, while the survey of Goethgebuer in the 1950’s recorded this species in more than one third of all sampling sites (**Figure 7**). These observations may indicate that the distribution of *Culicoides* species may change over the years depending on local ecological conditions.

**Annex D to Appendix 9**

**Studies on *Culicoides* found in association with livestock in the bluetongue virus (BTV) affected region of northern France**

*T. Baldet<sup>1</sup>, Jean-Claude Delecolle<sup>2</sup>*

<sup>1</sup>CIRAD, France, <sup>2</sup>University Louis Pasteur, Strasbourg, France

## Introduction

In the summer of 2006 northern Europe faced a very unusual situation due to the emergence of bluetongue (BT) disease in sheep and cattle. The first notification of BTV occurred in The Netherlands in the border area of Maastricht on August 14 and was confirmed by the Community Reference Laboratory at the Institute for Animal Health (IAH), Pirbright on August 17. Shortly thereafter, Belgium and Germany reported also outbreaks of BTV (on the 18th and on the 21<sup>st</sup>, respectively) and close to the same index area. Because of the risk of rapid extension by bluetongue the French government reinforced their monitoring program at the end of August.

In parallel with clinical and serological surveillance by DGAL/French Ministry of Agriculture, a preliminary entomological study was undertaken by CIRAD and the University Louis Pasteur de Strasbourg (ULP) and supported by the local veterinary services in north-eastern France (24-26 August). This preliminary transversal study was done to:

- verify the presence or absence of the Afro-asiatic *C. imicola*, which is the principal vector of BTV in the Mediterranean region;
- to collect qualitative and quantitative data on the local *Culicoides* fauna most notably on the Palaearctic species of the *C. obsoletus* group and/or of the *C. pulicaris* group, which, in the absence of *C. imicola*, have been implicated recently in outbreaks of BTV in southern Europe in the Balkans and in Sicily (Mellor and Wittmann, 2002; Torina et al. 2004; Purse et al. 2006); and
- to attempt to isolate BTV from wild-caught specimens of both vector groups as was achieved recently during the outbreaks of BTV in Italy (Savini et al. 2003; Carapacca et al. 2003; De Liberato et al. 2005). Furthermore, both species groups have been also experimentally infected with BTV-9 in the laboratory in the UK using field populations of *Culicoides* (Carpenter et al. 2006). Therefore, the role of the Obsoletus and Pulicaris species groups in the transmission of BTV is of real concern because they are common and widespread across the whole of central and northern Europe. Other local species, such as *C. nubeculosus* (**Figure 7**) have to be considered also as they occur abundantly near livestock.

On August 31, France reported an outbreak of BTV in the region of Ardennes close to the border with Belgium (and within the 150 km restriction zone that had been demarcated previously in response to the outbreaks in The Netherlands, Belgium and Germany). This case occurred in Brognon (49°55.46'N, 4°17.08'E) and involved a cow with slight clinical signs (lameness). On 2<sup>nd</sup> Sep two additional cases were notified: a clinically unaffected cow at Beaurieux (50°10.33'N, 4°07.38'E) and another at Hierges (50°06.19'N, 4°44.27'E). Both animals were detected in the framework of the BTV serological surveillance program. It is important to note that for each of these three French outbreaks all the remaining animals in the same herds tested negative for BTV, both in terms of virus detection and seroconversion. On 5 Sep 2006, France notified a 4th case of BTV in Ardennes: a bull calf with slight clinical signs (including salivation, nasal discharge, pyrexia and mouth congestion) at Tailly (49°26.06'N, 5°04.53'E). A further three cows from the same herd tested PCR positive but were serologically negative and showed no clinical signs.

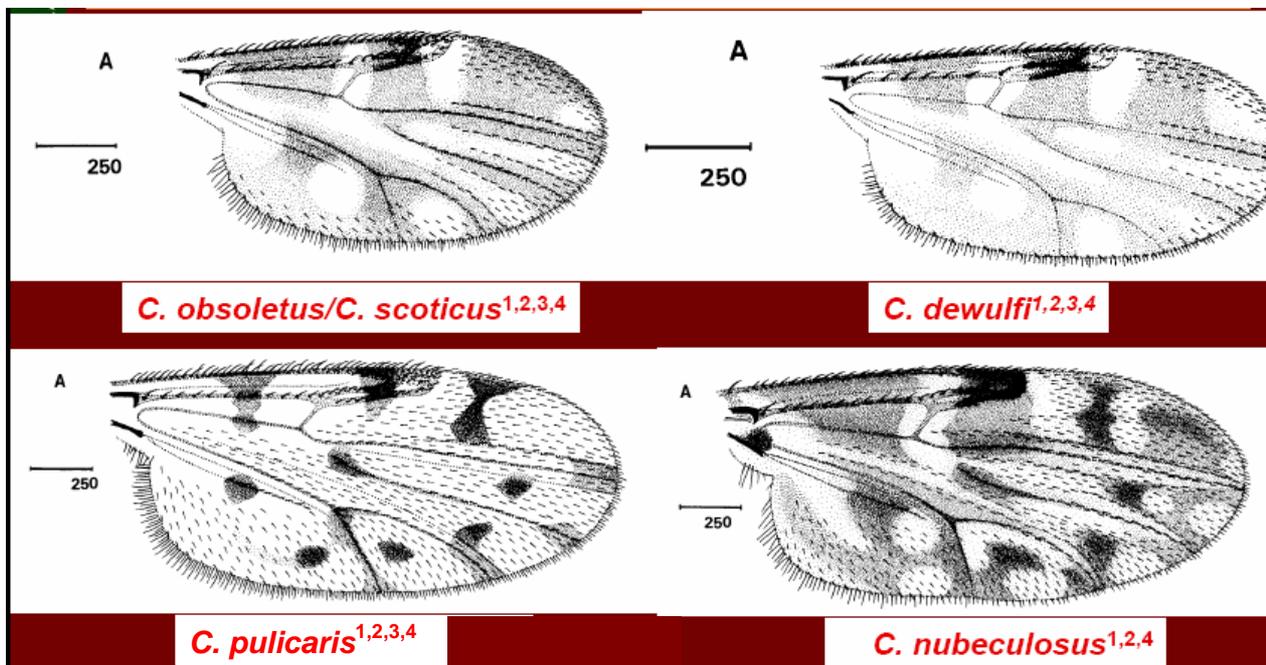
Simultaneously, a longitudinal entomological study was implemented in the affected area with the following objectives:

- to collect data on the spatial and temporal distribution of *Culicoides* in northern France (both on infected and on BTV-free farms) with emphasis on suspected local vectors i.e. the *C. obsoletus* group, the *C. pulicaris* group and *C. nubeculosus*, and
- to search for BTV in *Culicoides* trapped on infected farms to precisely identify the vector(s) involved.

BTV serotype 8 was identified by the IAH as the infecting virus in northern Europe. It has never been reported from Europe before and appears to have originated from sub-Saharan Africa. Although the disease was notified in August 2006, there is no doubt that BTV was already present in the index area prior to this date due to a lag between the date of its initial introduction and subsequent appearance in local livestock; this lag is a feature common to all vector-borne diseases (Gerbier et al. in press). The unexpected appearance of BTV at such a high northern latitude i.e. over 500 km further north than reported previously raises two important questions about the insect vectors:

- the supposed absence in northern Europe of the Afro-asiatic *C. imicola* the principal vector of BTV in Mediterranean basin, and
- if *C. imicola* is absent then what is the role played by Palaearctic species of *Culicoides* in the emergence of BTV so far north in Europe.

In this study the main results emanating from the entomological studies conducted in northern France are summarized and include both the transversal transect done before French outbreaks of BTV-8 appeared (24-26 August) and the longitudinal follow-up study of 13 consecutive weeks conducted in affected areas from the beginning of September to the end of November 2006.



**Figure 7. The BTV-8 outbreak in Northern France (2006): suspected *Culicoides* vectors**

- <sup>1</sup>: abundant on farms (according to season and environment).
- <sup>2</sup>: mammophilic (*C. pulicaris* is probably ornitho-mammophilic).
- <sup>3</sup>: found infected in outbreaks of BTV (*C. dewulfi* with BTV-8 in Gulpen, The Netherlands).
- <sup>4</sup>: competent vector of BTV (based on experimental laboratory infections).

## Materials and Methods

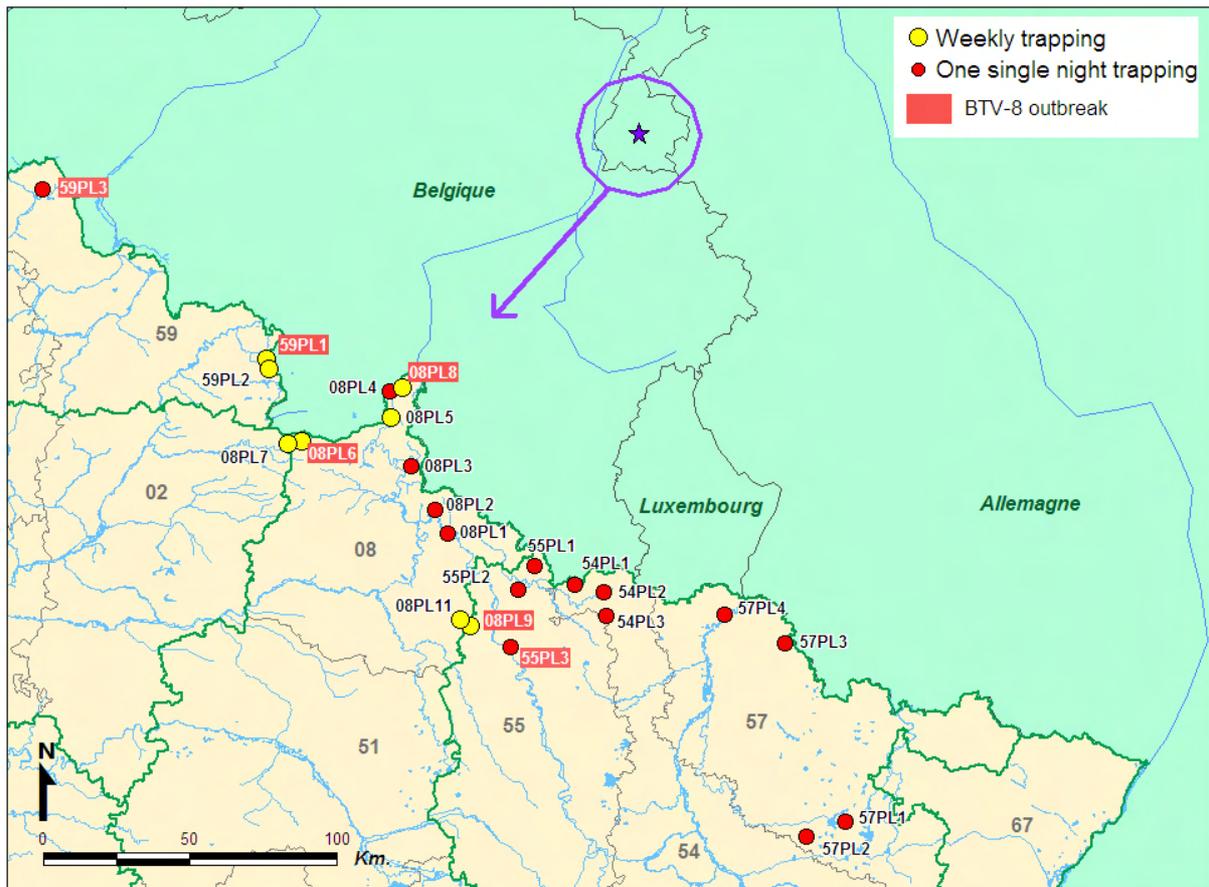
### Transversal transect (24-26 August)

Fourteen representative cattle and/or sheep farms located close to wetland and/or river sites were selected in north-eastern France (**Figure 8**): 12 along the frontier with Germany, Luxembourg and Belgium and two inland in a wet temperate area where some exotic *Culicoides* have been found in the past (such as *C. puncticollis* in 1982 and *C. shaklawensis* in 1995; Delécolle, pers. comm.). The

geographical co-ordinates and altitude of all trap sites were determined using a Garmin GPS 12 receiver. At each of these 14 sites, *Culicoides* were collected for a single night (seven sites on 24-25 August and seven on 25-26 August).

*Culicoides* were captured using Onderstepoort-type blacklight traps equipped with 8W UV-light bulbs and draught suction motors (**Figure 9**). Traps were operated from approximately one hour before sunset to one hour after sunrise; the traps were hung at 1.5-2 m above ground and were located outside buildings within 25 m of livestock. Insects attracted to the light were sucked into a plastic collection beaker containing 200–300ml of water, to which a few drops of detergent had been added to reduce surface tension. Large insects were excluded by mosquito netting (mesh size: 1–2 mm).

In the laboratory, and using a binocular microscope, all insects of the family Ceratopogonidae (including *Culicoides*) were first separated from all other insects. The *Culicoides* were identified based on wing pattern with identifications confirmed subsequently by mounting specimens on microscope slides (Delécolle 1985; Wirth and Marston 1968).



**Figure 8. Study sites in northern France (August – December, 2006)**  
**Transversal transect: single night trapping (24-26 August)**  
**Longitudinal follow up: weekly trapping (September to December)**

B. Longitudinal transect (weekly trapping from September to November)

Eight cattle and/or sheep farms were selected: four affected by BTV-8 (Brognon 08PL6, Beaurieux 59PL1, Hierges 08PL8, Taily 08PL9) and four paired BTV-free but located in a similar environment (respectively 08PL7, 59PL2, 08PL5, 08PL11).

Traps were operated one night per week under favourable climatic conditions (absence of heavy rain and/or wind) and outdoors (as described above) from week 36 (beginning of September) to week 48 (end of November) for 13 consecutive weeks. Contrary to the preliminary transect the field and laboratory methodologies differed slightly:

- The plastic collecting beakers of the OVI traps were filled with 200–300 ml of 70% ethanol (instead of water with a few drops of detergent added). The trapped insects were preserved in 70% ethanol at 4°C and are dispatched to the laboratory every two weeks maintaining the cold chain; 70% ethanol is recommended for preservation of BTV rDNA and for subsequent RT-PCR detection in infected midges (Melville et al. 2006).
- During morphological diagnosis in the laboratory, females of *Culicoides* were pooled according to site, date, species and physiological stage: (i) nulliparous, (ii) parous engorged, and (iii) parous non-engorged and preserved at 4°C. Detection of BTV was conducted by RT-PCR on monospecific pools of non-engorged parous females only. Parous females are

the older members of the *Culicoides* population and thus the ones most likely to harbour BTV.

If non engorged specimens prove to be BTV+ it is interpreted to reflect the presence of virus inside the insect i.e. represents an infection with BTV that can be transmitted subsequently to an animal host. These non-engorged specimens are separated from freshly engorged females which if BTV+ may simply reflect that they have imbibed a BTV+ blood meal but may not be capable of replicating BTV in the gut subsequently.



**Figure 9. OVI UV light trap:Gold standard method for sampling crepuscular and nocturnal biting *Culicoides* at livestock**

From week 39 onwards (end of September) weekly trappings were performed in parallel, and on the same night, both outdoors and indoors on 4 cattle farms in Ardennes; this part of the study was included in the longitudinal study (and used the same protocols described above). This additional study was done to compare indoor *versus* outdoor *Culicoides* biting activities since the external night temperatures drop sharply in winter and, in consequence, some animals are maintained inside the cattle sheds permanently. Three out of four farms were infected (08PL6, 08PL8, 08PL9) while one was BTV-free (08PL11).



**Outdoor**



**Indoor**

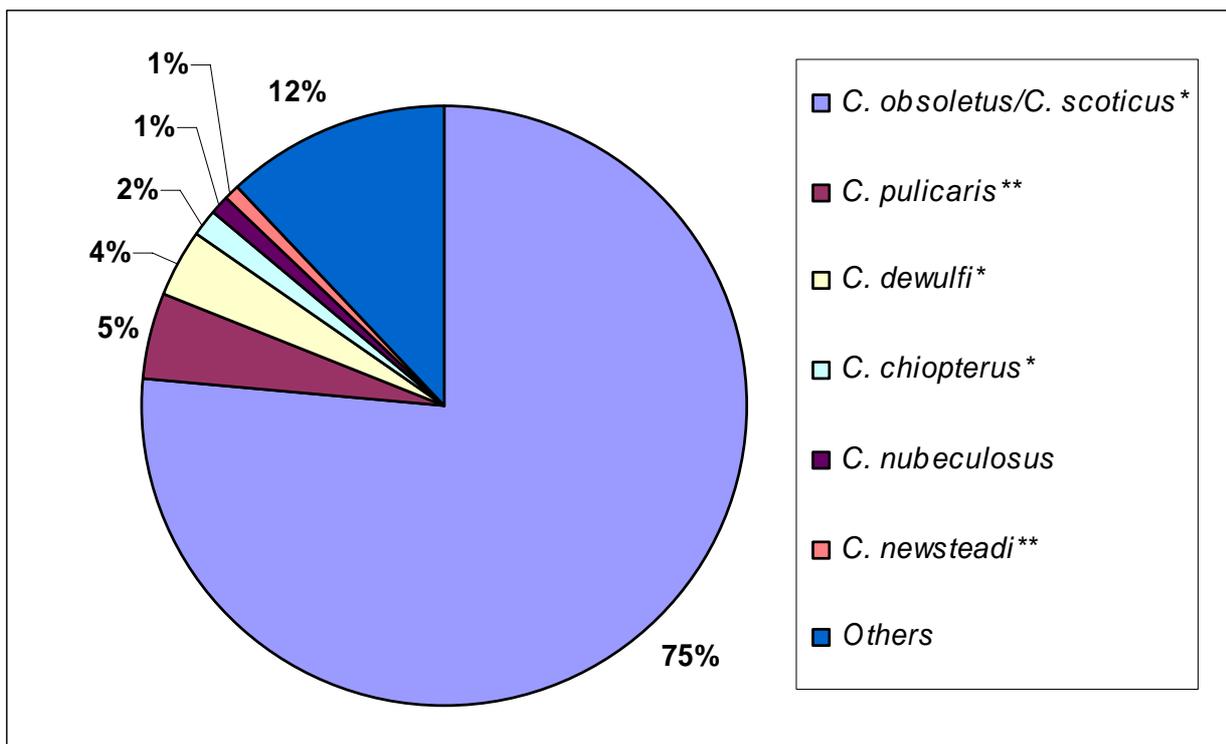
**Figure 10. Trapping of *Culicoides* outdoors and indoors on the same night at Hierges, a BTV-infected farm (08PL8)**

## Results

### Transversal transect (24-26 August)

The results are summarized in **Figure 11**. The important points are:

- Confirmed absence of *C. imicola* (the principal vector of BTV in southern Europe) and *C. impunctatus* (the “Scottish midge” abundant in some areas of northern Europe and during summer and autumn a serious nuisance biting to humans),
- A large diversity of *Culicoides* species were captured in the vicinity of livestock in north-eastern France with a high prevalence of *C. obsoletus/C. scoticus* (75%) followed by *C. pulicaris* (5%), *C. dewulfi* (4%), *C. chiopterus* (2%), *C. nubeculosus* (1%) and *C. newsteadi* (1%), with the exact species composition varying marginally between the sites. For example, *C. pulicaris* is absent in the lowland sites of the east but present in the forested mountains of the Ardennes region (France-Belgium border). Six other species were found at frequencies below 1% (*C. punctatus*, *C. festivipennis*, *C. lupicaris*, *C. stigma*, *C. salinarius* and *C. subfasciipennis*) and constituted 12% of all trappings made. It is to be noted that *C. obsoletus/C. scoticus*, *C. dewulfi* and *C. chiopterus* belong to the subgenus *Avaritia* and that *C. pulicaris*, *C. newsteadi*, *C. punctatus* and *C. lupicaris* belong to the subgenus *Culicoides* but more commonly referred to as the *C. pulicaris* group.
- Mean adult density was 42.6 *Culicoides*/trap/night. The minimum temperature recorded during these trapping nights was less than 5°C with a maximum temperature of <10°C.



**Figure 11. Relative prevalence of *Culicoides* species obtained from single night trappings in north-eastern France (24-25 August 2006)**

\**C. obsoletus* group

\*\**C. pulicaris* group

### Longitudinal follow up

Weekly sampling outdoors at eight sites (four BTV-infected and four BTV-free).

In 96 light trap collections >5,000 *Culicoides* were captured belonging to 18 species (**Table 2**). The catches were dominated by two species of the Obsoletus Complex (88.6%) namely *C. obsoletus* and *C. scoticus* (there are recorded together because the females of these two sibling species cannot be distinguished morphologically). The second-most abundant species was *C. pulicaris* (>4%); the third-most abundant taxon was *C. nubeculosus* (2.6%) followed by *C. dewulfi* (1.5%). These four species are suspected vectors of BTV (**Figure 7**) and provide the weekly variation in density for each species below. The remaining 14 species were found at frequencies below 1% and thus constituted only 3.2% of the total *Culicoides* captured. No specimens of *C. imicola* or of *C. impunctatus* were captured.

**Table 2. *Culicoides* species prevalence outdoors at eight sites (four BTV-infected and four BTV-free) in northern France (September to November 2006)**

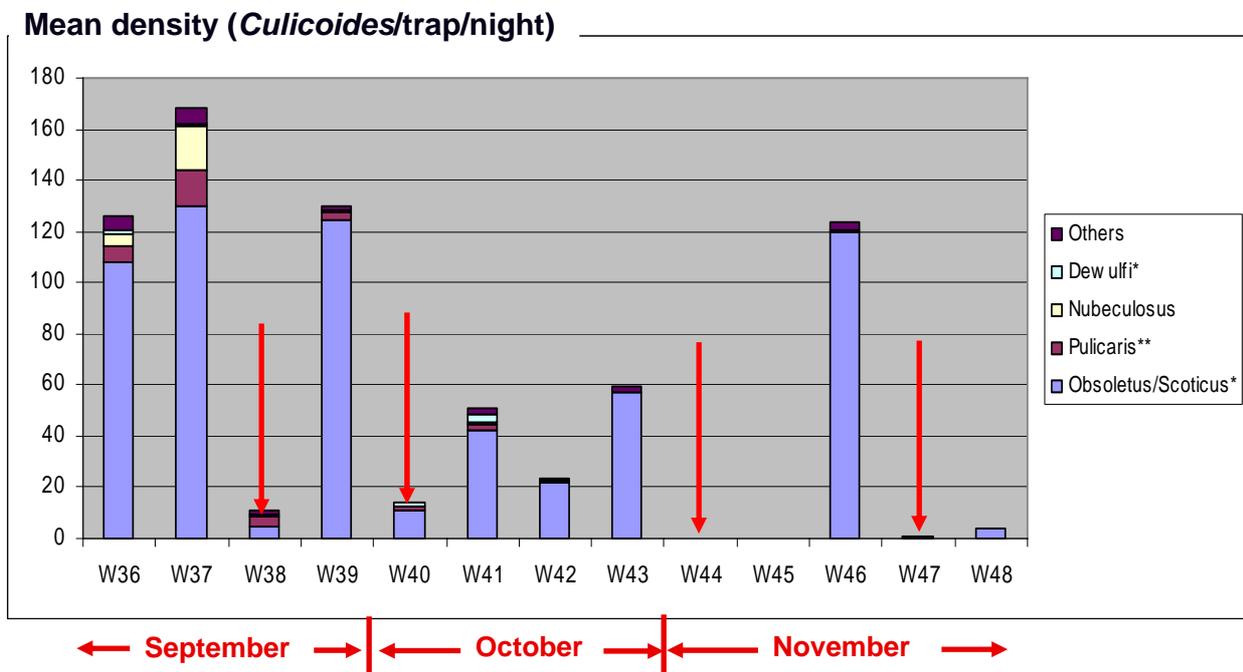
Range	Species	Number	Mean***	% total
1	<i>C. obsoletus/C. scoticus*</i>	4,506	46.9	88.6
2	<i>C. pulicaris**</i>	206	2.1	4.1
3	<i>C. nubeculosus</i>	133	1.4	2.6
4	<i>C. dewulfi*</i>	74	0.8	1.5
5	<i>C. chiopterus*</i>	47	0.5	0.9
6	<i>C. punctatus**</i>	37	0.4	0.7
7	<i>C. lupicaris**</i>	31	0.3	0.6
8	<i>C. salinarius</i>	15	0.2	0.3
9	<i>C. festivipennis</i>	14	0.1	0.3
10	<i>C. parroti</i>	5	0.1	0.1
11	<i>C. newsteadi**</i>	4	0.04	0.1
12	<i>C. deltus</i>	4	0.04	0.1
13	<i>C. circumscriptus</i>	2	0.02	0.04
14	<i>C. subfascipennis</i>	1	0.01	0.02
15	<i>C. riethi</i>	1	0.01	0.02
16	<i>C. achrayi</i>	1	0.01	0.02
17	<i>C. fascipennis</i>	1	0.01	0.02
18	<i>C. duddingstoni</i>	1	0.01	0.02
TOTAL		5,083	52.9	100.0

*C. obsoletus* group; \*\**C. pulicaris* group; both groups contain suspected vectors

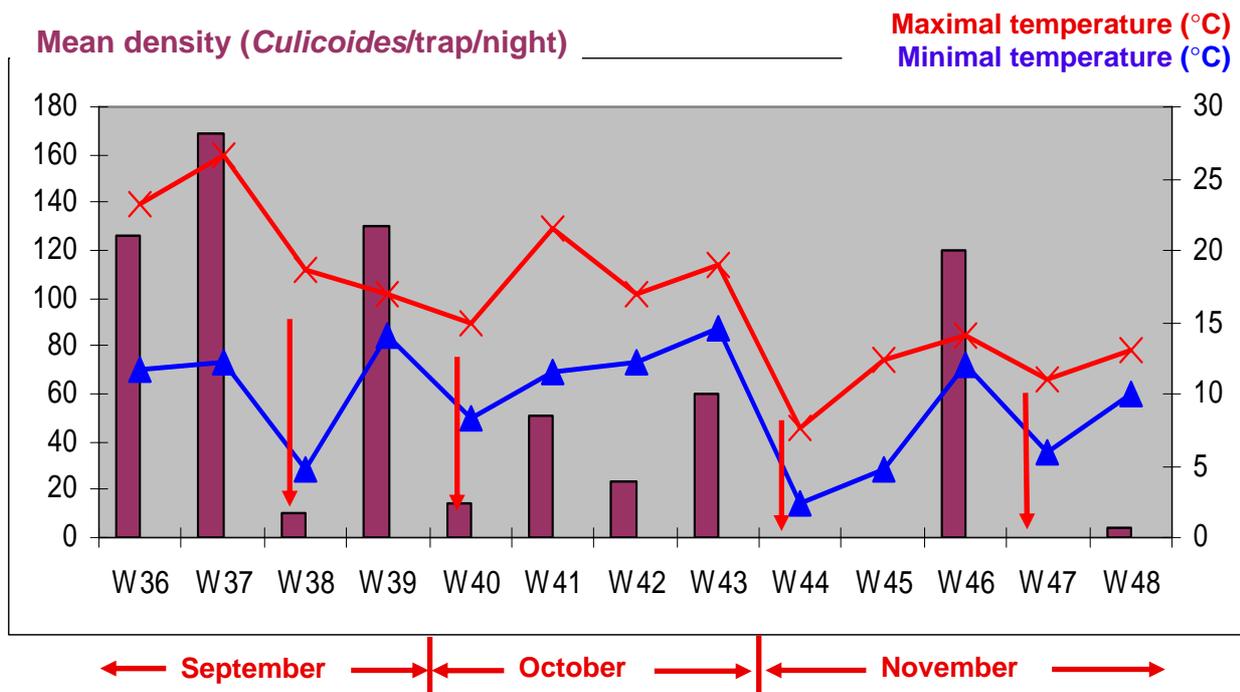
\*\*\*Mean density of *Culicoides*/trap/night (96 nights over 13 weeks; 5 September - 30 November)

**Figure 12** represents the weekly variation in mean density of *Culicoides* caught outdoors between September and November 2006. *C. obsoletus/C. scoticus* was predominant throughout autumn (September and October) and was quasi-exclusive in November. The *Culicoides* mean density outdoors is strongly related to daily external temperatures with no adult activity (based on the total absence of adult *Culicoides* in the light traps) when the maximum temperature was <10°C and the minimum temperature <5°C (**Figure 13**).

Notably *Culicoides* mean density in September (115.5 *Culicoides*/trap/night) is three times higher than at the end of August (42.6 *Culicoides*/trap/night). This result is confirmed when we compare the same site sampled at these two periods, for example 08PL5 with 477 *Culicoides* at the beginning of September (W36) as compared to 248 *Culicoides* at the end of August (W34). This is due to the very favourable weather conditions that prevailed from the beginning of September across Europe. Densities strongly decreased at the beginning of October (16.8 *Cul.*/t/n in W40) probably as a result of (i) the fall in night-time temperatures, and (ii) the housing of animals indoors.



**Figure 12. Weekly variation in *Culicoides* densities in northern France (Sept-Nov 2006)**  
**Outdoor** sampling during 13 consecutive weeks at eight sites (four BTV-infected and four BTV-free)



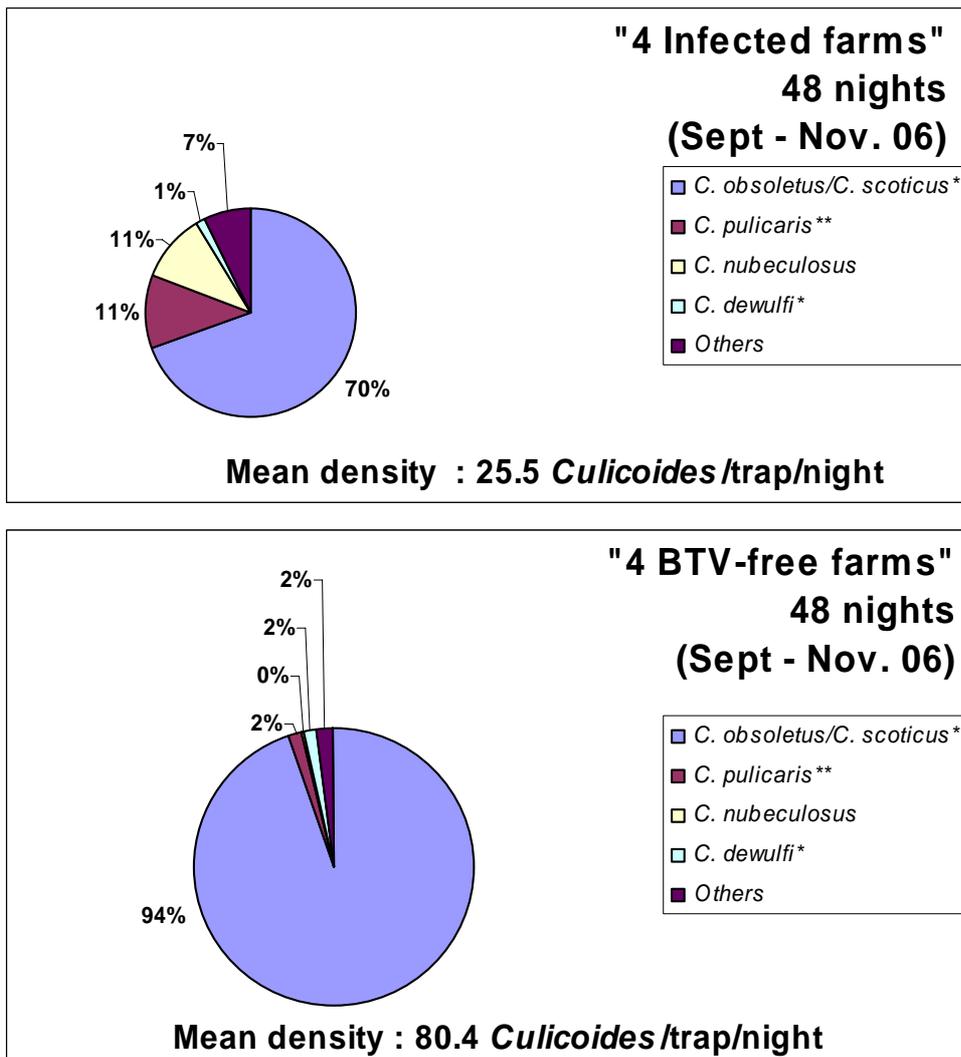
**Figure 13. Weekly variation in *Culicoides* densities related to temperature in northern France (Sept-Nov 06) - Outdoor sampling during 13 consecutive weeks at eight sites (four BTV-infected and four BTV-free)**

Importantly, it was found that even if outdoor *Culicoides* densities have decreased as a result of the drop in external temperatures, densities will increase again once temperatures rise (as shown in weeks 39, 41 and 46 (**Figure 13**)). However, despite the rise in temperature, densities do not rebound significantly after week 48 in late November. This is perhaps due to the cumulative effect and duration of cold periods and — directly linked to the temperature — the decline in the number of daylight hours. The reduction in length of the photoperiod has a direct impact on the number of hot hours and so could reduce (or even halt) larval development and lead to a total cessation in adult activity. To halt larval development — or to induce diapause — low day- and night-time temperatures are needed.

It is here necessary to distinguish between:

- the external night-time temperatures registered 2m above ground and influencing directly the active flight of adult *Culicoides* (taking into consideration that even if a very low minimum temperature is reached in the second part of the night, higher temperatures prevailing in the early hours of darkness will allow active flight by *Culicoides* adults), and
- the ground temperatures (day and night) influencing larval development in *Culicoides*. This can explain why a rise in temperature from one week to the next can lead to a rebound in adult populations.

A greater species diversity, but lower densities, of *Culicoides* were observed for the four infected farms in comparison to the four BTV-free farms (**Figure 14**). *C. dewulfi* (incriminated as a potential novel vector in Gulpen, The Netherlands, and presumed to be at least one of the vectors involved in the index area) was not abundant (1% on infected farms, 2% on BTV-free farms). The outbreak of BTV in France, during which the virus appears to have circulated little, raises questions about the local transmission of BTV-8.



**Figure 14. Comparison of relative outdoor prevalences of *Culicoides* species on ‘infected’ and ‘BTV-free’ farms in northern France (September to November 2006)**

*Weekly sampling on four farms (one trap outdoors and one trap indoors on each)*

Concerning the comparison of *Culicoides* biting activity indoors and outdoors, the results must be interpreted with caution since the buildings are not all open during the day and the night in the same way at the different sites. Also, the animal husbandry practises vary; on some farms a number of animals spend the night outside while on others they are all indoors. Nevertheless, overall *Culicoides* species diversity is lower indoors (six species) with nine species outdoors. In both locations, *C. obsoletus/C. scoticus* was dominant comprising 90.7% of the total caught outdoors and 95.0% indoors; *C. dewulfi* was less prevalent 3.2% outdoors and 2.1% indoors (**Figure 15**).

During the autumn the mean *Culicoides* densities indoors was 112.1 *Culicoides*/trap/night four times higher than outdoors (27.7) across a number of nights and under identical trapping conditions (**Table 3**) *Culicoides* will thus enter and be active inside livestock buildings in densities higher than those found outside.

**Table 3. Comparative indoor and outdoor *Culicoides* prevalences at four sites (three infected and one BTV-free) in northern France (October to December 2006)**

Range	Species	Number	Mean***	% total
1	<i>C. obsoletus/C. scoticus</i> *	956	25.2	90.7
2	<i>C. dewulfi</i> *	34	0.9	3.2
3	<i>C. pulicaris</i> **	24	0.6	2.3
4	<i>C. chiopterus</i> *	19	0.5	1.8
5	<i>C. punctatus</i> **	14	0.4	1.3
6	<i>C. newsteadi</i> **	2	0.1	0.2
7	<i>C. lupicaris</i> **	2	0.1	0.2
8	<i>C. duddingstoni</i>	2	0.1	0.2
9	<i>C. festivipennis</i>	1	0.03	0.1
<b>Total</b>		<b>1,054</b>	<b>27.7</b>	<b>100</b>

Outdoors

Range	Species	Number	Mean***	% total
1	<i>C. obsoletus/C. scoticus</i> *	4,049	106.6	95,0
2	<i>C. chiopterus</i> *	98	2.6	2,3
3	<i>C. dewulfi</i> *	91	2.4	2,1
4	<i>C. pulicaris</i> **	14	0.4	0,3
5	<i>C. punctatus</i> **	5	0.1	0,1
6	<i>C. achrayi</i>	3	0.1	0,1
<b>Total</b>		<b>4,260</b>	<b>112.1</b>	<b>100</b>

Indoors

\**C. obsoletus* group

\*\**C. pulicaris* group

\*\*\*Mean density *Culicoides*/trap/night (76 nights i.e. 38 outdoors and 38 indoors)

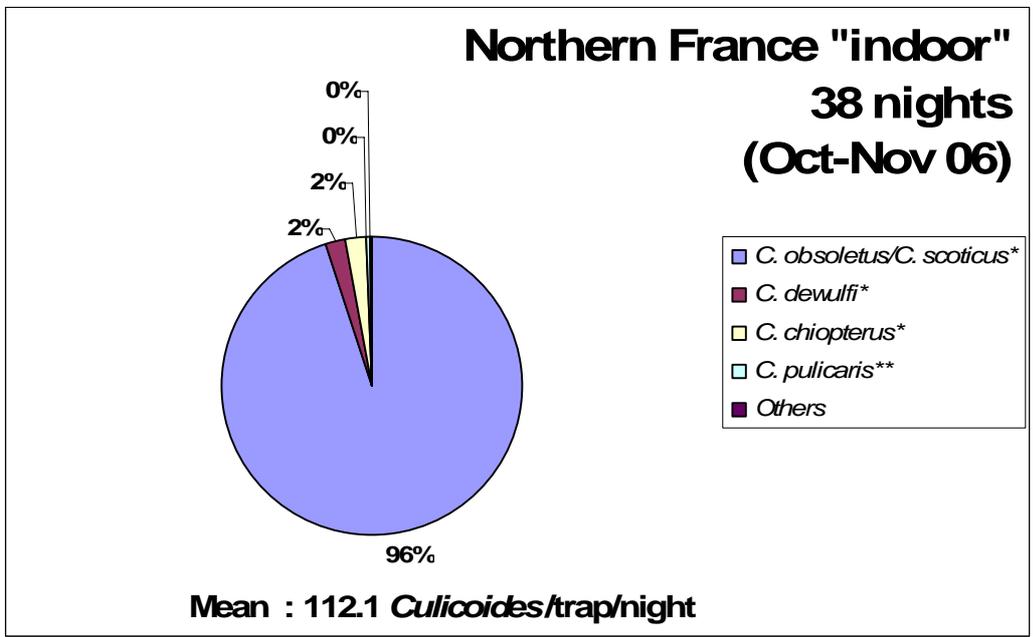
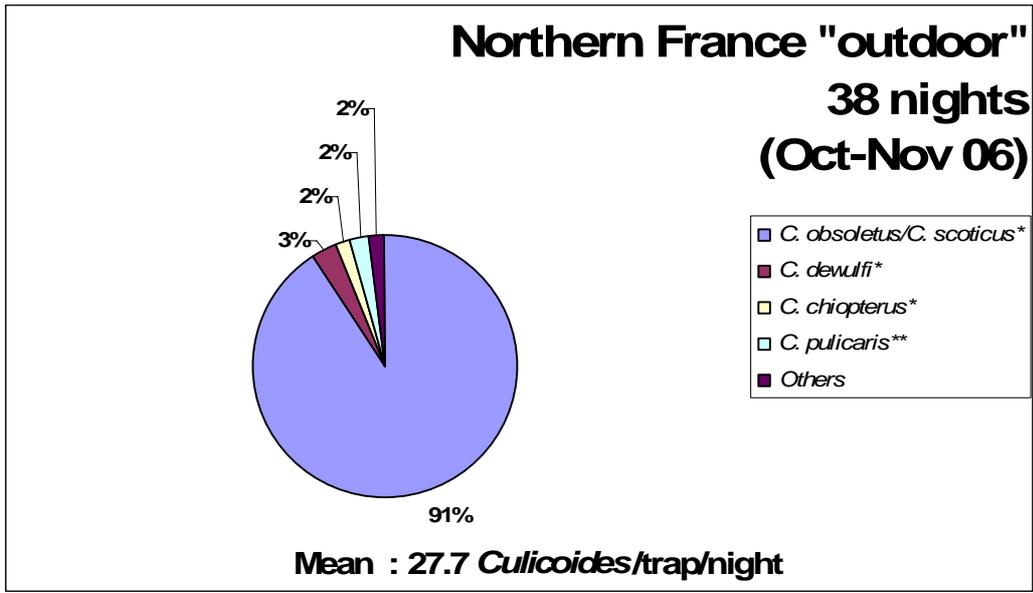
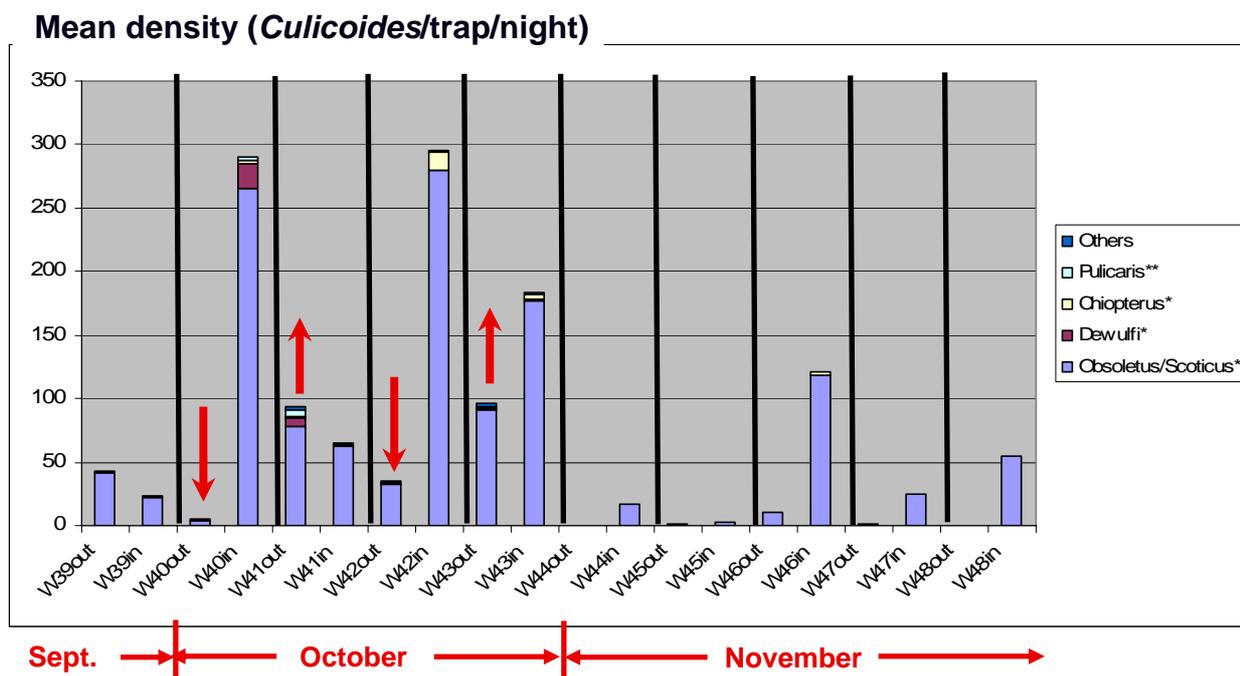


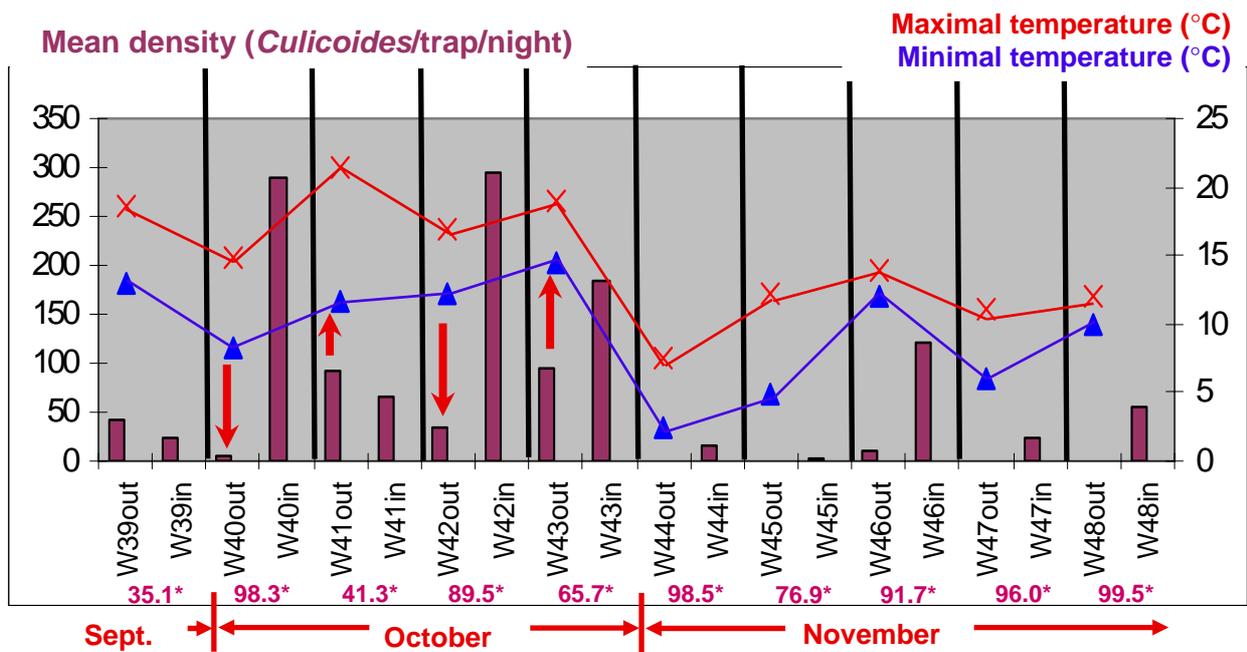
Figure 15. Comparative indoor and outdoor *Culicoides* prevalences in northern France (Oct - Nov. 2006)

Both outdoors and indoors *C. obsoletus/C. scoticus* was also predominant in October and quasi exclusive in November (Figure 16).



**Figure 16. Weekly variation of *Culicoides* densities both outdoors and indoors in northern France (Oct. to Nov. 2006) - Sampling on four farms (for each farm one trap outdoors and one trap indoors) W39 outdoors = Week39 outdoors - W39indoors = Week39 indoors**

The indoor trapping rate (ITR) seemed dependant on the outside temperature and not on the intrinsic behaviour of the insect (Figure 17). The ITR increased when external temperatures dropped and vice-versa. For example in W39, with a minimal temperature >10 °C and a maximal temperature >15 °C, the ITR was 35%. The following week (W40), with Tmin <10 °C and Tmax <15 °C, the ITR was 98% with only a few *Culicoides* caught outdoors. In W41, external temperatures increased (Tmin >10 °C and Tmax >15 °C) and the ITR dropped to 41%. Thus, the stalling of the animals inside buildings did not seem to be the most important factor contributing to this endophagic tendency since, between W40 and W41, the husbandry practices on the farm were not altered. At the beginning of November (W44) the weather was cold with Tmin <5 °C and Tmax <10 °C; no *Culicoides* activity was observed outdoors but a few specimens were still caught indoors (mean density <20 *Cul*/t/n). In Mid-November (W46) indoor biting activity increased due to a rise in the external temperature and reached a mean of 121 *Cul*/t/n against only 11 *Cul*/t/n outdoors. At the end of November temperatures were abnormally high for the season; *Culicoides* were still caught inside with a mean of 54 *Cul*/t/n while no *Culicoides* were captured outdoors. Mean *Culicoides* densities at the end of November were half those of mid-November despite external temperatures being relatively constant. As mentioned above, the cumulative effect and duration of cold periods as well as the reduction in the length of the photoperiod, probably lead to a drop in larval productivity and adult densities, both indoors and outdoors (even if the temperature rose for a short period during the last two weeks of November). This effect was more marked outdoors than indoors. This observation, plus the fact that we did not observe a significant difference between the outdoor and indoor *Culicoides* parous rate, leads us to conclude that there are no productive *Culicoides* breeding sites inside livestock buildings.



\*weekly indoor trapping rate

Figure 17. Weekly variation in outdoor and indoor *Culicoides* densities related to temperature, northern France (Oct. to Nov. 2006) - Sampling on four farms (for each farm one trap outdoors and one trap indoors) - W39outdoors = W39 outdoors and W39indoors = W39 indoors. Indoor trapping rate (ITR) = Total *Culicoides* caught Indoors/(Total Outdoors + Total Indoors) \*100

#### BTV diagnosis in *Culicoides*

A total of 3,127 non-engorged parous *Culicoides* females were captured and distributed amongst 119 pools (Table 4); 90% of the specimens belonged to *C. obsoletus*/*C. scoticus*. Half of these pools were tested. All proved to be BTV negative to RT-PCR.

**Table 4. BTV detection by RT-PCR in taxon-specific pools of non-engorged parous females, northern France (Sept - Nov. 2006)**

Outdoors	Pools	Number	Indoors	Pools	Number
<i>C. obsoletus/C. scoticus</i> *	34	1 054	<i>C. obsoletus/C. scoticus</i> *	25	1 763
<i>C. pulicaris</i> **	10	40	<i>C. dewulfi</i> *	6	49
<i>C. nubeculosus</i>	3	79	<i>C. chiopterus</i> *	10	87
<i>C. dewulfi</i> *	6	21	<i>C. pulicaris</i> **	5	6
<i>C. newsteadi</i> **	2	2	<i>C. punctatus</i> **	3	3
<i>C. punctatus</i> **	7	10	Total	49	1 908
<i>C. lupicaris</i> **	3	5			
<i>C. festivipennis</i>	3	3			
<i>C. circumscriptus</i>	1	2			
<i>C. parroti</i>	1	3			
Total	70	1 219			

\**C. obsoletus* group

\*\**C. pulicaris* group

## Discussion

### Relative prevalence of Culicoides found on livestock farms in autumn in northern France

Out of the 9,343 *Culicoides* caught in the study area, none were *C. imicola* (the main vector of BTV in southern Europe including the Mediterranean Basin); nor was it found in light trap collections made across The Netherlands, Belgium and Germany during the same period. Although *C. imicola* appears not to have established itself in northern Europe, this does not exclude the remote possibility of BTV-8 having been brought into the region by a single infected individual of *C. imicola* (by airplane during the transport of animals and/or plants).

*C. impunctatus*, the Scottish biting midge, earlier suspected to be a potential vector of BTV because of its abundance in preliminary studies in The Netherlands, and because of its mammophilic biting preferences, was not detected either. However, the Palaearctic species suspected to be potential vectors of BTV in Europe, mainly *C. obsoletus/C. scoticus* and, to a lesser extent *C. pulicaris*, were present.

*C. nubeculosus*, which was shown (Wittmann, 1999) competent at transmitting BTV in the laboratory at temperatures ranging between 30 °C-35 °C, was captured also. *C. nubeculosus* is mammalophilic and can be found in large numbers in northern Europe. It is one of the few species of *Culicoides* than can be colonised successfully in the insectary, which facilitates studies on its vector competency through experimental infections.

*C. dewulfi*, found RT-PCR+ to BTV-8 in Gulpen (The Netherlands), is rare on farms in northern France, including those affected by BTV-8; it comprised <3% of the total *Culicoides* captured both indoors and outdoors, which is far lower than its overall total of 17% in Gulpen. In mid-September

(W37), the mean density of *C. dewulfi* in northern France was 0.5/trap/night (out of a total mean of 168 *Cul/t/n*); its mean density at Gulpen during the same period was 58/t/n (for a total mean of 343 *Cul/t/n*). Trapping protocols (OVI trap), study period, weather conditions (temperature, rainfall) and morphological diagnostic skills were comparable in both situations.

This marked difference in the relative abundances of *C. dewulfi* between France and The Netherlands could be explained by environment and the activities of man as both influence the presence and productivity of favourable breeding sites. Most of the studied sites in northern France were located in the hilly and forested Ardennes region, whereas Gulpen comprises mostly open lowland pastures interspersed amongst mature stands of deciduous forest. *C. dewulfi* is known to breed in cow dung lying naturally in the field (Kettle and Lawson 1952). This dependence on bovid dung means that the distribution of *C. dewulfi* will be determined almost totally by that of cattle. Cattle density, breeding practices, soil composition, and relief have to be more fully investigated, both in northern France and in the region of Gulpen, to better understand the differences. The results of the trapping campaigns carried out simultaneously in other regions in The Netherlands, Germany and Belgium (in particular in the Ardennes) will be very informative especially in regard to the mean density and the relative prevalence of each *Culicoides* species based on the environment and husbandry practises.

A clear understanding of *Culicoides* systematics and taxonomy is crucial to virtually all bluetongue virus (BTV) vector studies. The most important *Culicoides* vectors exist as species complexes (such as *C. obsoletus* and *C. pulicaris* groups) and the members of these complexes may occur sympatrically or allopatrically. Since individual members may differ widely in vectorial capacity it is vital that they be distinguished to understand exactly which species within a complex plays a vectorial role. Consequently, the risk of transmission may vary dramatically at the national level, depending upon the particular cryptic species present in a given area.

In northern Europe most researchers in virus isolation or vector competency studies are not able to confidently distinguish *C. dewulfi* from other sympatrically caught species of the Obsoletus Complex i.e. *C. obsoletus*, *C. scoticus* and *C. chiopterus*. Accurate identification, a sound taxonomy, is fundamental to such studies and exposes the lack of taxonomic expertise currently available in Europe (Meiswinkel et al. 2004). Recently developed PCR-specific tools will be helpful in defining both the vectorial role and larval biotopes of these sibling species in Europe (Mathieu et al. in press).

#### Seasonal distribution of *Culicoides* found near livestock in autumn in northern France

The epidemiology of BTV and other *Culicoides*-borne orbiviral diseases is linked strongly to climate and weather and can influence the spatial and seasonal dynamics of its vectors. For example, in temperate epizootic areas such as in the Mediterranean Basin, BT emerges usually during the late summer and autumn when vector population are large and composed mainly of old females (whose age is compatible with BTV replication and transmission).

In northern France it was observed at the beginning of September 2006, and under good climatic conditions, that *Culicoides* populations increased in comparison to those encountered at the end of August. The low temperatures and relatively heavy rains which characterised the climate in northern Europe in August 2006 likely had a negative impact on the populations of livestock-associated *Culicoides* inducing a decline in larval productivity (flowing water disrupting breeding sites, a decrease in the egg hatching rate and slower development of the larval stages) and suppressed also adult activity. Another consequence is that lower temperatures are known to also decrease the competence of some vectors to transmit BTV (Mullens et al. 1995; Paweska et al. 2004). Thus the unfavourable climatic conditions prevailing in August could have led to a decline in the vector population and of virogenesis in the vector resulting in a reduced viral transmission

potential; this could — in part — explain the interrupted incidence in outbreaks of BTV noted two weeks later (mid-September) and causing its unusual bimodal pattern.

Subsequently, the exceptional climatic conditions prevailing thereafter in northern Europe — particularly in October — facilitated the prolonged maintenance of BTV (with the epizootic peaking in mid-October). However, in November, the number of outbreaks of BTV began to fall just when temperatures fell also and when the size of vector populations decreased; despite these suppressant events BT disease did not disappear. This lag is explained (in part) by the length of viral incubation in the vertebrate host (before the appearance of clinical signs 1-2 weeks later) and an additional lag in laboratory confirmation and official declaration of the outbreak (another week). In northern Europe the outbreak of BTV-8 was contained only two weeks or more following the first frosts of the winter season.

Following extended fluctuations — in all probability linked to the rise in temperatures in mid-November — the decline in the incidence of BTV-8 in Belgium, Germany and the Netherlands was sharply noticeable, reflecting decreased temperatures (and the subsequent decrease in vector activity) but only at the beginning of December. Some outbreaks were declared also after this date but is likely due to residual infected midges remaining active in favourable locales.

In northern France the vector-free period was declared to commence in mid-December after the two previous weeks of light trapping had yielded <10 *Culicoides*/trap/night both outdoors and indoors (but with some residual *Culicoides* activity — notably indoors — on some farms) reflecting the cold advance of winter. It remains to be seen whether the disease will disappear totally during the coming winter months to not resurface the following year (late summer 2007). There is a fear that bluetongue could become endemic in northern Europe and that it could recrudescence in the post-winter season (cf. comments below).

#### Indoor biting activity and overwintering of livestock *Culicoides* in northern France

It is assumed falsely that *Culicoides* are purely exophagic and exophilic i.e. that they feed only on animals outdoors and will not enter and/or rest in buildings. In northern France it was observed in October and November that adult *Culicoides* activity was higher indoors than outdoors. This endophagic tendency seemed to be controlled principally by the external temperatures and, secondarily, by animal husbandry practises (stalling of the animals inside buildings). In South Africa thousands of *C. bolitinos* are known to enter stables; and if all farm animals are housed each night it will likely 'force' *Culicoides* to enter cattle sheds and stables to suck blood (Meiswinkel et al. 2000). It is thus imperative to follow indoor biting activity all year round and to link the findings to climatic factors (temperature and rainfall) and to husbandry practises (including shed typology).

Another false belief is that *Culicoides* are strictly nocturnal. Most species are also a nuisance during the day displaying two biting peaks: one after sunrise the other close to sunset. But these biting hours lengthen when overcast conditions prevail leading to biting throughout the day in darkened environments. Diurnal *Culicoides* activity patterns inside cattle sheds should be studied.

The intensity of daytime attacks (which include those by *C. obsoletus*) is deserving of more entomological research because it has implications for the diurnal transmission of BTV. The use of animal baits for biting rate studies is recommended if we wish to explore further the contact between *Culicoides* and cattle both night and day; this is because the classical method of sampling use light traps is limited during the daytime (even if the light trap remains the gold standard for collecting data on the geographic distribution and seasonal dynamics of *Culicoides*).

The lack of productive *Culicoides* breeding sites within livestock buildings is reassuring but needs to be confirmed through additional studies. The advent of colder temperatures — in particular

freezing temperatures for a prolonged period — should definitively interrupt larval production (by inducing hibernation) and halt all adult activity (even inside cattle sheds).

The longevity of adult *Culicoides* has not been measured precisely; likely it lasts from a few weeks (mostly) to many months (rarely). Adult *Culicoides*, especially in more temperate areas, will find winter conditions too harsh for them to survive until the following season. Thus in colder regions, most *Culicoides* species overwinter in the egg or in the larval stage in diapause.

Should BTV manage to survive — either in a vector or in a susceptible host throughout the winter — fresh outbreaks should be expected next year once adult vector populations begin to increase in spring. In regard to the overwintering of BTV in *Culicoides* two possibilities exist:

- the cross-winter persistence of infected residual adult *Culicoides* populations, and/or
- vertical transmission of BTV in *Culicoides*.

It is unlikely that infected competent adult *Culicoides* will survive the winter but the possibility that they may be able to persist inside cattle sheds (where a more favourable temperature regime may occur) must be researched. Although the S7 genome segment of BTV has been detected in overwintering *C. sonorensis* larvae in the near vicinity of outbreak sites in northern Colorado (USA) during a long-term field study (White et al. 2005), there is no published evidence to suggest that any *Culicoides*-transmitted virus, and among them BTV, can be passed vertically (transovarially or venereally) through the vector, unlike many others vector-transmitted arbovirus (Culicidae, Phlebotomidae, ticks). Several laboratory studies have failed to reveal the existence of vertical transmission (Jones and Foster, 1978; Nunamaker et al. 1990; Fu et al. 1999).

#### The epidemiological context in France

The extremely low levels of BTV-8 circulation in France could be due to:

- its late mid-August introduction when *Culicoides* populations were decreasing because of the unfavourable weather, and/or
- isolated infections perhaps due to only a few infected midges being dispersed erratically from the more northerly infected zone (via long range north-south passive wind dispersal along the Meuse River and/or its tributaries) without initiating intensive local transmission.

The lack of intensive local transmission can perhaps be linked to the near absence of the newly suspected BTV-8 vector *C. dewulfi* in the north of the Ardennes. If the environment and/or husbandry practices in the Ardennes region do not favour *C. dewulfi* it would be of interest to conduct similar studies in the adjoining region of Belgium where the number of outbreaks was also far lower than encountered further north. Notwithstanding other factors (such as the reduced dispersal of infected midges southwards and a lower regional cattle density) the hypothesis linking reduced BTV circulation to significantly smaller populations of *C. dewulfi* needs to be explored further.

The implication of *C. dewulfi* as a vector of BTV-8 in The Netherlands should not be interpreted to exclude other *Culicoides* from involvement in the north European outbreaks. It would be prudent to consider the Obsoletus group (mainly *C. obsoletus* and *C. scoticus*) and more secondarily *C. pulicaris* as having played also a vectorial role not only because they have been implicated in southern Europe but also because they are consistently abundant in northern Europe (as was for *C. obsoletus/C. scoticus* and — to a lesser extent — *C. pulicaris* at Gulpen).

Although additional studies are required to more conclusively implicate *C. dewulfi* as a vector of BTV it now seems abundantly clear that *Culicoides* endemic to northern Europe are quite capable of replicating and transmitting the virus and that they can do so without the ‘help’ of *C. imicola*. This vectorial capacity of European *Culicoides* will likely increase under the influence of a globally warming climate.

## Conclusion

The emergence of BTV-8 in The Netherlands, Belgium, Luxemburg, Germany and France has led to a significant development in the epidemiology of the disease in the EU. This serotype of BTV had never been encountered before anywhere in Europe. From the entomological point of view future research should be focussed on the following aspects:

- improving *Culicoides* taxonomy, especially that dealing with sibling species complexes, using both morphological and molecular tools, with the one method informing the other;
- studying the vector competency of Palaearctic *Culicoides* using experimental laboratory infections (which includes identifying molecular markers for distinguishing vector from non-vector species — most notably for those species which are difficult to colonise);
- investigating the possible vertical transmission of BTV in vector *Culicoides*;
- developing methods and tools for measuring vector capacity, particularly in a field context and taking into account species-specific interactions with the environment;
- understanding more thoroughly the ecology of *Culicoides* (characterization of breeding sites, adult dispersal both local and regional, seasonal dynamics, adult overwintering) and their determinants (environment and animal husbandry practices);
- developing methods of vector control and evaluating their efficacy in terms of disease reduction and/or seroconversion;
- creating predictive models based on environmental and ecological patterns that could help identify areas — and seasons — at risk to incursions by BTV;
- investigating modifications in the epidemiology of BTV under differing scenarios of climate change.

## Acknowledgements

This work was funded by the DGAL/French Ministry of Agriculture and conducted by CIRAD-EMVT in collaboration with the University Louis Pasteur de Strasbourg. We would like to thank Dr Catherine Cêtre-Sossah for BTV diagnosis in *Culicoides* and Dr Guillaume Gerbier and Mr Rudy Meiswinkel for helpful comments and suggestions. For their support in the field we thank the local Veterinary Services, in particular François Ricatte (DDSV 57), Jean-Jacques Maret (DDSV 54), Maurice Deiller (DDSV 55), Jean-Charles Quintard, Anne Bronner, Bénédicte Thomas (DDSV 08), Annick Pinard (DDSV 51) and JP Verhellen (Private veterinarian S/C DDSV 51).

## References

- Caracappa S, Torina A, Guercio A, Vitale F, Calabro A, Purpari G, Ferrantelli V, Vitale M and Mellor PS 2003. Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. *Vet. Rec.* 153 (3): 71-74.
- Carpenter S, Lunt HL, Arav D, Venter GJ and Mellor PS 2006. Oral susceptibility to bluetongue virus of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *J Med Entomol.* 43 (1): 73-78.
- De Liberato C, Scavia G, Lorenzetti R, Scaramozzino P, Amaddeo D, Cardeti G, Scicluna M, Ferrari G and Autorino GL 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. *Vet Rec.* 156 (10): 301-304.
- Delécolle J-C 1985. Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. Thèse d'Université. Université Louis Pasteur de Strasbourg UER Sciences Vie et Terre.
- Fu H, Leake CJ, Mertens PPC and Mellor PS 1999. The barriers to bluetongue virus infection, dissemination and transmission in the vector, *Culicoides variipennis* (Diptera: Ceratopogonidae). *Arch. Virol.* 144: 747-761.
- Gerbier G, Biteau-Coroller F, Grillet C, Parodi J, Zientara S, Baldet T, Guis H and Roger F 2006. A description of the outbreak of Bluetongue (BTV 4) in Corsica in 2003: lessons for surveillance. *Vet Rec.* (in press).
- Jones RH and Foster NM 1978. Heterogeneity of *Culicoides variipennis* field populations to oral infection with bluetongue virus. *Am. J. Trop. Med. Hyg.* 27, 178-183.
- Kettle DS and Lawson JWH 1952. The early stages of British biting midges *Culicoides* Latreille (Diptera: Ceratopogonidae) and allied genera. *Bull. Ent. Res.* 43: 421-467.
- Mathieu B, Perrin A, Baldet T, Delécolle J-C, Albina E and Cetre-Sossah 2007. Molecular identification of the Western European species of the *Obsoletus* complex (Diptera: Ceratopogonidae) by an ITS-1 rDNA multiplex PCR assay. *J. Med. Ent.* (in press).
- Meiswinkel R, Baylis M and Labuschagne K 2000. Stabling and the protection of horses from *Culicoides bolitinos* (Diptera: Ceratopogonidae), a recently identified vector of African horse sickness. *Bull. Ent. Res.* 90 (6): 509-515.
- Meiswinkel R, Gomulski LM, Delécolle J-C, Goffredo M and Gasperi G 2004. The taxonomy of *Culicoides* vector complexes – unfinished business. *Vet. Ital.* 40 (3): 151-159.
- Mellor PS and Wittmann EJ 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. *Vet. J.* 164: 20-37.
- Melville L, Hunt N, Bellis G and Harmsen M 2006. Improving bluetongue virus surveillance in remote areas Cairns. Australia: ISVEE 11.
- Mullens BA, Tabachnick WJ, Holbrook FR and Thompson LH 1995. Effects of temperature on virogenesis of Bluetongue virus serotype 11 in *Culicoides variipennis sonorensis*. *Med. Vet. Ent.* 9: 71-76.

- Nunamaker RA, Sieburth PJ, Dean VC, Wigington JG, Nunamaker CE and Mecham JO 1990. Absence of transovarial transmission of bluetongue virus in *Culicoides variipennis*: immunogold labeling of bluetongue virus antigen in developing oocytes from *Culicoides variipennis* (Coquillett). *Comp. Biochemist. Physiol.*, 96: 19-31.
- Paweska JT, Venter GJ and Mellor PS 2004. Vector competence of South African *Culicoides* species for bluetongue virus serotype 1 (BTV-1) with special reference to the effect of temperature on the rate of virus replication in *C. imicola* and *C. bolitinos*. *Med. Vet. Ent.*, 16: 10-21.
- Savini G, Goffredo M, Monaco F, De Santis P and Meiswinkel R 2003. Transmission of bluetongue virus in Italy. *Vet. Rec.* 152: 119.
- Torina A, Caracappa S, Mellor PS, Baylis M and Purse BV 2004. Spatial distribution of bluetongue virus and its *Culicoides* vectors in Sicily. *Med. Vet. Ent.* 18: 81-89.
- White DM, Wilson WC, Blair CD and Beaty BJ 2005. Studies on overwintering of bluetongue viruses in insects. *J. Gen. Virol.* 86: 453–462.
- Wirth WW and Marston N 1968. A method for mounting small insects on microscope slides in Canada balsam. *Ann. Entomol. Soc. Am.* 61: 783-784.
- Wittmann EJ 2000. Temperature and the transmission of arboviruses by *Culicoides* biting midges. PhD Thesis. University of Bristol, Bristol.