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Culicoides monitoring in Belgium in 2011: analysis of spatiotemporal abundance, species diversity and Schmallenberg virus detection

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Abstract. In 2011, *Culicoides* (Diptera: Ceratopogonidae) were collected at 16 locations covering four regions of Belgium with Onderstepoort Veterinary Institute (OVI) traps and at two locations with Rothamsted suction traps (RSTs). Quantification of the collections and morphological identification showed important variations in abundance and species diversity between individual collection sites, even for sites located in the same region. However, consistently higher numbers of *Culicoides* midges were collected at some sites compared with others. When species abundance and diversity were analysed at regional level, between-site variation disappeared. Overall, species belonging to the subgenus *Avaritia* together with *Culicoides pulicaris* (subgenus *Culicoides*) were the most abundant, accounting for 80% and 96% of all midges collected with RSTs and OVI traps, respectively. *Culicoides* were present during most of the year, with *Culicoides obsoletus* complex midges found from 9 February until 27 December. Real-time reverse-transcription polymerase chain reaction screening for Schmallenberg virus in the heads of collected midges resulted in the first detection of the virus in August 2011 and identified *C. obsoletus* complex, *Culicoides chiopterus* and *Culicoides dewulfi* midges as putative vector species. At Libramont in the south of Belgium, no positive pools were identified.

Key words. *Culicoides*, midges, monitoring, Schmallenberg virus, vector.

Introduction

Biting midges are small haematophagous Diptera belonging to the family Ceratopogonidae, genus *Culicoides*. Some species in this genus are known vectors of arboviruses like African horse sickness virus, Akabane virus (AKAV), bluetongue virus (BTV), epizootic haemorrhagic disease virus and equine encephalosis virus (Mellor *et al.*, 2000; Johnson *et al.*, 2012).

Immediately after the start of the BTV serotype 8 (BTV-8) outbreak in 2006 in central and northern Europe, several countries initiated *Culicoides* monitoring programmes to study the abundance, composition, dispersal and seasonal dynamics of these insects and to define vector-free periods (De Deken *et al.*, 2008; Meiswinkel *et al.*, 2008a, 2008b; Casati *et al.*, 2009; Hoffmann *et al.*, 2009; Hörbrand & Geier, 2009; Kiel *et al.*, 2009; Vorsprach *et al.*, 2009; Berisha *et al.*, 2010; Silbermayr

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Table 1. *Culicoides* trapping locations used in Belgium in 2011.

Trap type	Location	Livestock	Trap position	Longitude, ° E	Latitude, ° N
OVI trap	Antwerp				
	Nijlen	Dairy cows	Stable	4.693747	51.159744
	Nijlen	Horses	Stable	4.687219	51.155171
	Varendonk	Dairy cows	Stable	4.954160	51.085820
	Kessel	Horses, cattle, sheep, chickens	Meadow	4.607064	51.154562
	Berlaar	Dairy cows	Stable	4.665713	51.118610
	Olen	Cattle	Stable	4.865726	51.151850
	Eindhout	Horses	Stable	4.972531	51.086183
	Betekom	Sheep, deer, chickens	Meadow	4.79206	51.00200
	Liège				
	Verlaine	Dairy cows	Meadow	5.379914	50.611208
	Boncelles	Horses	Stable	5.554803	50.567739
	Sart Tilman	Cattle, sheep, goats, chickens, horses	Stable	5.587336	50.576544
	Nandrin	Horses, cattle, pigs	Stable	5.358419	50.528464
	Bettincourt	Chickens	Meadow	5.236917	50.712872
	Gembloux	Cattle, sheep, pigs	Stable	4.72662	50.56509
	Libramont				
	Libramont	Sheep	Meadow	5.35956	49.92881
	Libramont	Cattle	Meadow	5.35636	49.92931
RST	Gembloux	None	Meadow	4.70942	50.56294
	Libramont	Cattle, sheep	Meadow	5.36082	49.92667

OVI, Onderstepoort Veterinary Institute; RST, Rothamsted suction trap.

et al., 2011; Ander *et al.*, 2012). Furthermore, midges collected during surveys conducted around this period were used to identify putative vector species, which led to the detection of BTV-8 in *Culicoides obsoletus* complex, *Culicoides dewulfi*, *Culicoides chiopterus* and *Culicoides pulicaris* (De Liberato *et al.*, 2005; Savini *et al.*, 2005; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007; Dijkstra *et al.*, 2008; Vanbinst *et al.*, 2009). The oral susceptibility of *Culicoides scoticus* and vector competence of *Culicoides sonorensis* for BTV-8 have also been demonstrated (Carpenter *et al.*, 2008a; Veronesi *et al.*, 2013a).

In 2011, a new orthobunyavirus called Schmallenberg virus (SBV) (Hoffmann *et al.*, 2012), which causes mild symptoms in adult cattle but severe congenital malformations in lambs, calves and goat kids, was identified. This virus spread rapidly over Europe after its emergence (Conraths *et al.*, 2013; Doceul *et al.*, 2013; European Food Safety Authority, 2013; Chaintoutis *et al.*, 2014). *Culicoides* midges were suspected to be responsible for this fast spread of SBV because they are known vectors for several related viruses (e.g. AKAV) (St George *et al.*, 1978; Al-Busaidy & Mellor, 1991; Bryant *et al.*, 2005; Yanase *et al.*, 2005). Several studies using field-collected midges have detected SBV in *C. obsoletus* complex, *C. chiopterus* and *C. dewulfi* midges (De Regge *et al.*, 2012; Rasmussen *et al.*, 2012; Goffredo *et al.*, 2013; Elbers *et al.*, 2013a, 2013b; Larska *et al.*, 2013a, 2013b) and a vector competence study has shown the replication and dissemination of SBV in *C. sonorensis* (Veronesi *et al.*, 2013b).

In the present study, *Culicoides* collected at 16 sites dispersed over four regions in Belgium during 2011 were used to analyse potential differences in species abundance and diversity between individual collection sites and among regions. Furthermore, the collected midges were used to study several aspects of the epidemiology of SBV.

Materials and methods

Culicoides trapping

In 2011, *Culicoides* were collected with Onderstepoort Veterinary Institute (OVI) traps operated with 12-V batteries (Venter *et al.*, 2009) at 16 different locations (Table 1) covering four regions of Belgium (Fig. 1), including Antwerp (northeast), Liège (east), Gembloux (central) and Libramont (south). During January and February and July–September, *Culicoides* were collected every second week, whereas during March–June and October–December, weekly catches were performed. Collections were carried out on 41 collection dates. Numbers of collections at each location are shown in Table 2. Each collection with a blacklight trap comprised one night. Insects were retained in 60% ethanol. All OVI traps were installed outside in the immediate vicinity of stables (< 20 m) or in meadows in which animals were present throughout the year (Table 1).

Culicoides were also collected at two sites [Gembloux and Libramont (Table 1)] with stationary Rothamsted suction traps (RSTs) (Macaulay *et al.*, 1988) capturing *Culicoides* at a height of 12 m. Trapping occurred continuously during 2011 and the traps were emptied almost daily (363 times at Gembloux and 323 times at Libramont). Trapped insects were stored in 80% ethanol.

Morphological identification

The biting midges were morphologically identified to species level under the microscope using the key of Delécolle (1985) and stored in 80% ethanol. *Culicoides obsoletus sensu strictu* (*C.obsoletus s.s.*) and *C. scoticus* collected at Antwerp were

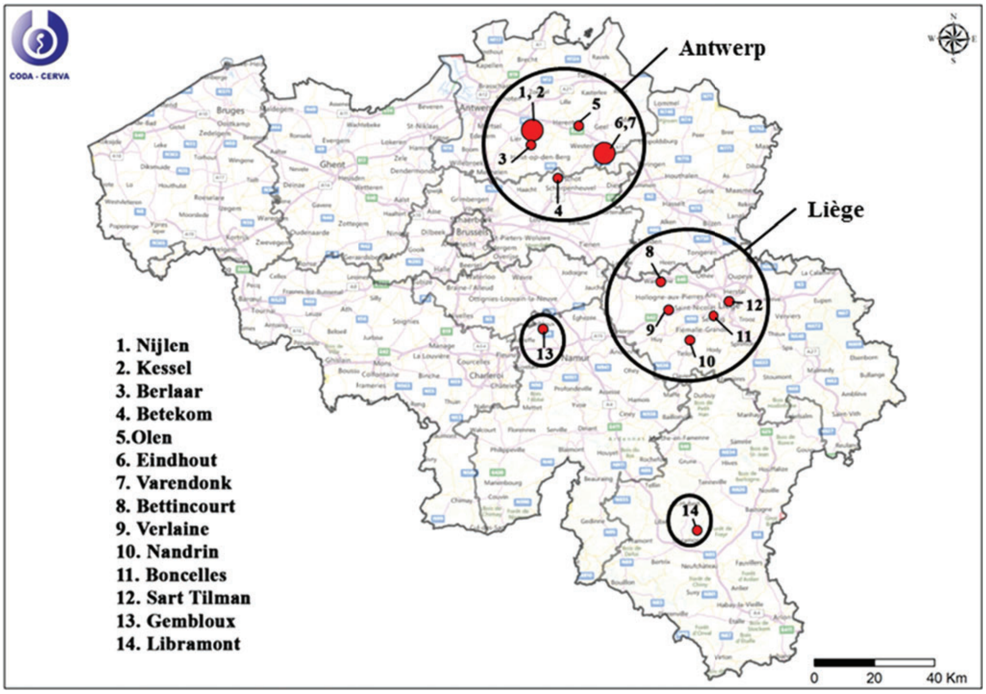

Fig. 1. *Culicoides* trapping locations in Belgium used during monitoring in 2011.

identified at species level, whereas collections of these two species at Liège, Gembloux and Libramont were grouped in the *C. obsoletus* complex. If collections exceeded 200 midges, 200 *Culicoides* were randomly selected and identified and numbers were extrapolated to the entire catch.

rRT-PCR analysis of pools of Culicoides

Species-specific pools containing a maximum of 25 heads of non-engorged parous females collected between July and October 2011 at Antwerp and Liège were tested for the presence of SBV. A similar approach was used for *Culicoides* collected between August and November at Gembloux and Libramont. Some additional pools of heads of blood-engorged female *Culicoides* were included.

The pools were analysed for the presence of the S and L segments of SBV by real-time reverse-transcription polymerase chain reaction (rRT-PCR) as described previously (De Regge *et al.*, 2012). Pools positive for one or both segments were considered SBV-positive in the calculation of infection prevalence. To ensure that any proposal of putative vector species based solely on rRT-PCR data was conservative, only species in which both the S and L segments were detected were considered as putative vectors.

Statistical analysis

To determine whether certain locations or regions were more prone than others to support higher numbers of *Culicoides* collected, a ranking was made for each collection date whereby the location or region where the highest (mean) number of *Culicoides* were collected received the highest rank. This was done for all dates for which collections were available for each site or region and average rankings were calculated. Friedman's test was used to determine whether differences between ranks were significant. If significant differences were found, two-by-two comparisons of the locations were carried out using Wilcoxon post hoc tests. Bonferroni correction to compensate for the number of two-by-two comparisons was applied.

After the ranking of mean catch sizes per month for each location within a particular region, similar analyses were performed to determine whether the probability of collecting greater numbers of *Culicoides* was higher in particular months.

Differences between the ratios of SBV-positive pools collected at individual locations in the region of Liège were assessed using Fisher's exact test.

Statistical analyses were performed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, U.S.A.). P values < 0.05 were considered to be significant.

Results

Monitoring with OVI traps

Abundance and seasonality. A total of 382 000 *Culicoides* were collected over 41 separate collection dates in 2011 using 16 OVI traps (Table 2). High variations in abundance were observed at each location throughout the year. Ranking of the different locations by abundance for each collection date showed

Table 2. *Culicoides* abundances in 2011 in four regions of Belgium based on monitoring with 16 Onderstepoort Veterinary Institute (OVI) traps and two Rothamsted suction traps (RSTs).

Trap type	Region	Location	Total number collected, *n*	Collections, *n*	Catch, mean ± SD	Average intra-region rank	Average inter-region rank based on mean catch
OVI	Antwerp		Total 58 105		184 ± 652		2.5^{a}
		Nijlen	1824	38	48 ± 87	3.2^{a}	
		Varendonk	15 001	40	375 ± 1415	5.0^{b}	
		Kessel	460	40	12 ± 25	2.7^{a}	
		Berlaar	12 180	40	305 ± 603	6.0^{b}	
		Nijlen, manege	1096	40	27 ± 76	2.9^{a}	
		Olen	7209	40	180 ± 372	5.0^{b}	
		Eindhout	3591	40	90 ± 186	5.3^{b}	
		Betekom	16 744	38	441 ± 845	6.0^{b}	
	Liège		Total 218 854		1128 ± 3097		3.2^{b}
		Verlaine	6453	40	161 ± 285	2.4^{a}	
		Boncelles	47 534	40	1188 ± 2324	3.7^{b}	
		Sart Tilman	58 979	40	1474 ± 2612	3.6^{b}	
		Nandrin	102 865	37	2780 ± 5741	3.6^{b}	
		Bettincourt	3023	37	82 ± 153	1.8^{a}	
	Gembloux		20 407	38	537 ± 1356		1.9^{a,b}
	Libramont		Total 84 661		1209 ± 4171		2.4^{a,b}
		Libramont–sheep	66 942	35	1913 ± 5771	1.9^{b}	
		Libramont–cow	17 719	35	506 ± 994	1.1^{a}	
RST	Gembloux		1137	363	3 ± 5	—	—
	Libramont		6410	323	20 ± 31	—	—

Locations or regions with the same superscript letter did not significantly differ in two-by-two comparisons using Wilcoxon post hoc tests subsequent to findings of significant differences using Friedman's test.
SD, standard deviation.

Berlaar and Betekom to have the highest average rankings in the region of Antwerp (Friedman's test, $P < 0.0001$). No significant differences in the probability to collect high numbers between these sites and Olen, Eindhout and Varendonk were found. In contrast, the probability to collect high numbers was lower at Nijlen, Nijlen Manege and Kessel. At Liège (Friedman test, $P < 0.0001$), the collection sites of Boncelles, Sart Tilman and Nandrin provided significantly higher probabilities to collect high numbers than Verlaine and Bettincourt. When a similar analysis was done based on mean catch sizes of each region, significant differences in average ranking were found (Friedman test, $P < 0.0001$) and two-by-two comparisons showed that the probability to collect high numbers was significantly higher in Liège than in Antwerp. Mean numbers per collection (Fig. 2) were clearly higher in the months of April–November than during December–March, indicating that the probability of obtaining a more abundant catch is higher during spring, summer and autumn. This is reflected in the higher average ranking of these months in a ranking of mean catch size by month (data not shown). Despite the reported extensive individual variation, high numbers of *Culicoides* were collected in all four regions during weeks 19 and 34 (data not shown).

Species diversity. Forty *Culicoides* species were identified in the OVI trap collections during 2011 (Table 3). *Culicoides obsoletus* and *C. scoticus*, which together accounted for 86% of individuals, were the most abundant species to be collected in Belgium. Other prominent species were *C. dewulfi* (5%) and *C. chiopterus* (3%), making the *Avaritia* the dominant subgenus, covering 94% of all individuals. *Culicoides pulicaris* (2%) was also relatively abundant. The other 35 species together accounted for about 4% of the total collection.

Important variation in species diversity was found between the individual collection sites (Table 3). Although *C. obsoletus/scoticus* represented the most prominent species at all locations, their relative abundance ranged from 38% to 96%. Interestingly, some species that were absent or present in low numbers at most locations were highly abundant at others. For example, *Culicoides kibunensis* accounted for 31% of collections at Verlaine, *Culicoides punctatus* represented 10% of midges caught at Nijlen and *Culicoides nubeculosus* represented 9% of midges trapped at Betekom. However, when species diversity was examined at regional level (Table 3), relative abundances did not differ significantly between regions.

Monitoring with RST traps

Using RSTs, totals of 1137 and 6410 *Culicoides* were collected at Gembloux and Libramont, respectively, in 2011 (Table 2). In total, 29 species were collected by RSTs (Table 4). Except for *Culicoides riouxi*, all species were also collected with OVI traps. A clear difference in species composition between RST and OVI trap collections performed at nearby sites was observed (Table 3). Whereas *C. obsoletus* complex midges were

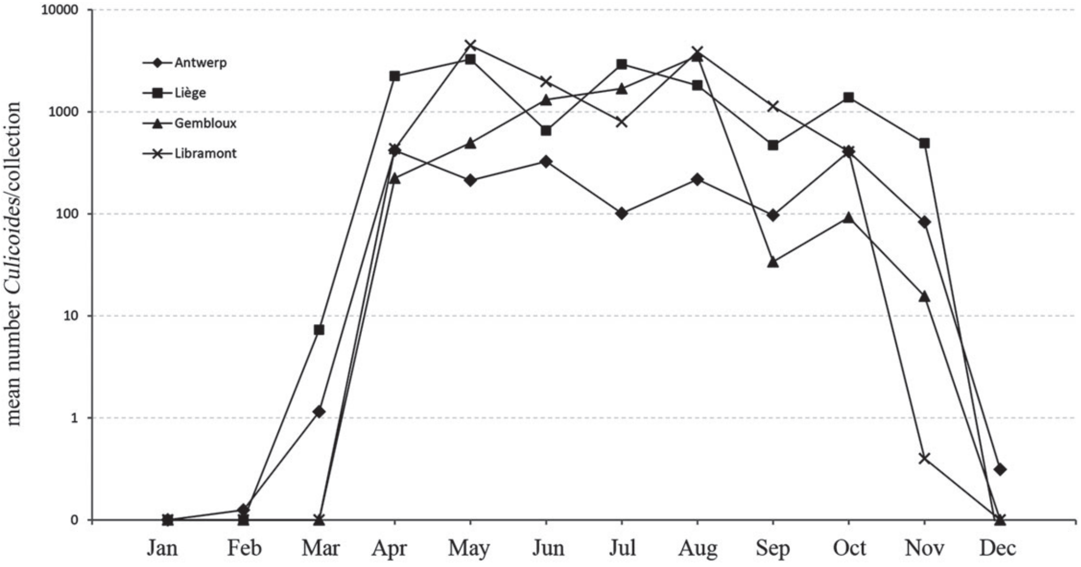

Fig. 2. Monthly spatiotemporal abundances of *Culicoides* in 2011 based on monitoring with 16 Onderstepoort Veterinary Institute (OVI) traps covering four regions of Belgium. *Culicoides* were monitored in Antwerp, Liège, Gembloux and Libramont with eight, five, one and two OVI traps, respectively. The mean number of *Culicoides* per collection is based on maximums of two (January, February, July, August, September), four (April, May, October, December) or five (March, June, November) collection dates in the respective month.

relatively less abundant in RST collections than in OVI trap collections (54% vs. 80% and 35% vs. 92% in Gembloux and Libramont, respectively), *C. pulicaris* and *C. chiopterus* were relatively more abundant, indicating that species were more evenly distributed. Further, more species had relative abundances of > 1% in RST collections, certainly at Libramont.

Phenology

Species belonging to the subgenus *Avaritia* and some belonging to the subgenus *Culicoides* (*C. pulicaris*, *C. punctatus*, *Culicoides lupicaris*) were present during most of the year, ranging from periods of 7 or 8 months (April/May to November) to 11 months for *C. obsoletus* complex midges (Table 4). Although quite wide variation was observed for other species, most midges belonging to the subgenera *Oecacta* and *Wirthomyia* were found only for short periods, ranging from 1 week to 2 months (mostly in May and June).

When the presence of *Culicoides* across the different regions as determined by OVI trap collections is considered, it is apparent that the dates of first appearance are earliest in the northeast (15 February at Eindhout) and progress towards the east (1 March at Boncelles), the centre (31 March at Gembloux) and the south [5 April at Libramont (sheep)] (Table 5). In the central and southern regions, activity also seemed to stop a little earlier (mid-November) than in the northeast and east regions (end of November and beginning of December, respectively). At Betekom in the region of Antwerp, low numbers of *C. obsoletus/scoticus* were collected until the end of December (27 December).

Similarly to the OVI traps, the first and last species collected with both RSTs were *C. obsoletus* complex midges. *Culicoides* were collected with RSTs during a timeframe similar to those of OVI traps located in the near vicinity (Table 5), except that the last *Culicoides* were trapped at Gembloux on 6 December with an RST and on 8 November with an OVI trap. At Libramont, a single *C. obsoletus* complex midge was collected on 9 February, but the next individual was not collected until 30 March.

Schmallenberg virus detection in pools of Culicoides heads

The distribution of species-specific pools analysed for the presence of SBV reflected the species diversity of OVI trap collections. Most of the pools ($n = 331$) consisted of *C. obsoletus/scoticus*, followed by other species belonging to the *Avaritia* [*C. dewulfi* ($n = 41$ pools) and *C. chiopterus* ($n = 42$ pools)]. Furthermore, several pools ($n = 31$) of *C. pulicaris* and a limited number of pools ($n = 35$) of non-abundant species were analysed (Fig. 3). A total of 480 pools representing 7305 midges were tested.

Some pools containing heads of *C. obsoletus*, *C. dewulfi* and *C. chiopterus* midges were found to be positive for both the S and L segments of SBV and these species are therefore proposed as putative vector species for SBV. Only one pool of *C. pulicaris* was found positive for the S segment (Ct = 37.9); positivity was not confirmed for the L segment.

The first S segment-positive pool was collected in the region of Liège (Boncelles) on 10 August and consisted of *C. obsoletus* complex midges (Table 6, Fig. 3). Of the pools collected in the same region, a further three of 58 collected in August and three of 39 pools collected in September were found to be S segment-positive; all of these consisted of *C. obsoletus* complex midges. This number increased substantially in October, with

Table 3. Species diversity and relative abundances (%) in *Culicoides* collections in Belgium in 2011 obtained with 16 Onderstepoort Veterinary Institute (OVI) traps and two Rothamsted suction traps (RSTs).

		Antwerp									Liège						Gembloux		Libramont			
		Nijlen	Varen-donk	Kessel	Berlaar	Nijlen, stable	Olen	Eind-hout	Bete-kom		Ver-laine	Bon-celles	Sart Tilman	Nandrin	Bettin-court				Sheep		Cow	
	Overall ∗	OVI	OVI	OVI	OVI	OVI	OVI	OVI	OVI	**Total**∗	OVI	OVI	OVI	OVI	OVI	**Total**∗	OVI†	RST†	OVI†	RST†	OVI	**Total**∗
C. obsoletus/scoticus	**86**	77	78	51	73	66	78	85	81	**78**	38	96	86	90	56	**88**	80	54	92	35	68	**87**
C. pulicaris	**2**	1						2	2	**1**	10	2	1	1	4	**2**	7	11	1	28	7	**2**
C. chiopterus	**3**	4	6	3	9		17		2	**6**	1		3	3	18	**2**	3	21	2	10	10	**4**
C. dewulfi	**5**	5	12	10	16	21	4	7	3	**9**	5		8	3	15	**4**	4	5	3	5	7	**4**
C. kibunensis				9							31				4	**1**	1	1		4		
C. subfasciipennis				1							1									1		
C. heliophilus																				1		
C. festivipennis				3							4									1		
C. punctatus		10	2	6		7		3		**1**	1				1		1	3				
C. nubeculosus									9	**3**												
C. newsteadi																	3					
C. lupicaris																		1		2		
C. stigma																		2		9		
Others	**4**	3	2	17	2	6	1	3	3	**2**	9	2	2	3	2	**3**	1	2	2	4	8	**3**

∗Indicates overall, total or mean values obtained with OVI traps.

†The OVI trap and the RST were separated by 1.25 and 0.25 km at Gembloux and Libramont, respectively.

Table 4. Location and time of first and last appearances of species identified during the *Culicoides* monitoring of 2011. *Culicoides* were collected with 16 Onderstepoort Veterinary Institute (OVI) traps and two Rothamsted suction traps (RSTs) in four regions of Belgium.

Subgenus	*Culicoides* species	OVI	RST	First capture date, 2011	First capture place	Last capture place	Last capture date, 2011
Avaritia	*C. chiopterus*	x	x	02/04	Gembloux RST	Varendonk stable	13/12
	C. dewulfi	x	x	13/04	Sart Tilman	Gembloux RST	19/11
	C. obsoletus and *scoticus*	x	x	07–09/02	Libramont RST	Betekom	27/12
Beltranmyia	*C. circumscriptus*	x	x	19/04	Betekom	Nandrin	04/11
	C. manchuriensis	x	—	19/04	Betekom	Olen	24/08
	C. salinarius	x	x	21/04	Verlaine	Sart Tilman	05/10
Culicoides	*C. deltus*	x	x	26/04	Libramont	Bettincourt	04/11
	C. grisescens	x	—	15/06	Berlaar	Nijlen	28/06
	C. impunctatus	x	x	10/05	Libramont, Nijlen, Kessel, Varendonk stable	Boncelles, Bettincourt	10/08
	C. lupicaris	x	x	07/04	Gembloux RST	Gembloux	08/11
	C. newsteadi	x	x	19/04	Varendonk	Varendonk	12/10
	C. pulicaris	x	x	23/03	Nandrin	Boncelles, Nandrin	18/11
	C. punctatus	x	x	29/03	Varendonk	Bettincourt	30/11
Monoculicoides	*C. nubeculosus*	x	x	13/04	Bettincourt	Varendonk, Betekom	12/10
	C. parroti	x	x	21/06	Gembloux RST	Bettincourt	05/10
	C. puncticollis	x	—	15/06	Varendonk	Varendonk	20/09
	C. riethi	x	x	10/05	Bettincourt	Varendonk	12/10
	C. stigma	x	x	07–14/04	Libramont RST	Libramont RST	04–07/11
Oecacta	*C. alazanicus*	x	—	30/05	Verlaine	Varendonk stable, Betekom	24/08
	C. albicans	x	—	19/04	Varendonk	Nandrin	30/05
	C. albihalteratus	x	—	21/04	Verlaine	Verlaine, Sart Tilman, Nandrin	30/05
	C. brunnicans	x	x	19/04	Berlaar, Betekom	Libramont RST	10/06
	C. clastrieri	x	—	15/07	Verlaine	Verlaine	15/07
	C. festivipennis	x	x	26/04	Varendonk, Betekom	Bettincourt	04/11
	C. furcillatus	x	—	10/05	Sart Tilman	Varendonk	12/10
	C. heliophilus	x	x	10/05	Sart Tilman, Libramont RST	Libramont RST	20–23/07
	C. kibunensis	x	x	21/04	Verlaine	Verlaine	25/09
	C. pictipennis	x	x	04/04	Berlaar	Verlaine	22/06
	C. poperinghensis	x	—	19/04	Berlaar	Verlaine	22/06
	C. simulator	x	—	18/05	Verlaine	Verlaine	30/05
	C. truncorum	x	—	30/05	Sart Tilman, Nandrin	Sart Tilman, Nandrin	30/05
	C. vexans	x	x	14/04	Gembloux RST	Gembloux	21/06
Silvaticulicoides	*C. achrayi*	x	x	28/04	Sart Tilman	Varendonk, Varendonk stable	02/11
	C. fascipennis	x	x	07/06	Libramont	Libramont RST	15/09
	C. pallidicornis	x	x	08/05	Libramont RST	Libramont	23/08
	C. picturatus	x	—	30/05	Verlaine, Sart Tilman	Bettincourt	28/10
	C. subfasciipennis	x	x	10/05	Verlaine, Sart Tilman, Kessel	Libramont RST	27/08
Wirthomyia	*C. minutissimus*	x	x	06/05	Libramont RST	Gembloux RST	11/07
	C. riouxi	—	x	27/05	Libramont RST	Libramont RST	14/06
	C. segnis	x	x	26/04	Libramont	Libramont	23/08

24 of 48 pools found to be S segment-positive (19 pools of *C. obsoletus* complex, four pools of *C. dewulfi* and one pool of *C. chiopterus*). In the region of Antwerp, four (three of *C. obsoletu*s and one of *C. pulicaris*) of 36 pools collected in September and one (*C. dewulfi*) of 68 pools collected in October were found to be positive for the S segment. In Gembloux, two of 14 pools collected in October were S segment-positive. In the south of Belgium, at Libramont, no positive pools were found, indicating a lower circulation of SBV.

The Ct values for the rRT-PCR detecting the S segment varied between 26.6 and 38.5 in the positive pools (Table 6). Most of the pools with a Ct value of > 35 for the S segment were not found positive for the L segment by rRT-PCR.

An analysis of data for the region of Liège, where 31 S segment-positive pools were found during August–October 2011, showed that none of the individual locations seemed more suitable than others for collections of SBV-positive midges (Fisher's exact test, $P = 0.559$) (Table 7).

Discussion

The *Culicoides* survey of 2011 showed that midges belonging to the subgenus *Avaritia* (*C. obsoletus/scoticus*, *C. dewulfi*, *C. chiopterus*), together with *C. pulicaris* (subgenus *Culicoides*), represent the most abundant and widely distributed species

Table 5. Weeks of first and last *Culicoides* collections in Belgium in 2011 obtained using 16 Onderstepoort Veterinary Institute (OVI) traps and two Rothamsted suction traps (RSTs).

	Antwerp									Liège						Gembloux		Libramont			
	Nijlen OVI	Varendonk OVI	Kessel OVI	Berlaar OVI	Nijlen, stable OVI	Olen OVI	Eindhout OVI	Betekom OVI	Mean*	Verlaine OVI	Boncelles OVI	Sart Tilman OVI	Nandrin OVI	Bettincourt OVI	Mean*	OVI †	RST †	Sheep OVI †	RST †	Cow OVI	Mean*
First detection (week)	13	13	14	13	12	13	7	11	**12**	13	9	12	11	14	**12**	14	13	14	6‡	16	**15**
Last detection (week)	45	47	46	51	45	45	51	52	**48**	46	48	49	46	48	**47**	45	49	47	47	42	**45**

*Indicates overall, total or mean values obtained with OVI traps.

†The OVI trap and the RST were separated by 1.25 and 0.25 km at Gembloux and Libramont, respectively.

‡This represents a single *Culicoides obsoletus* complex midge collected on 9 February; the next individual was not collected until 30 March.

in Belgium and are, moreover, present for most of the year. These results are in agreement with findings in other European countries (Casati *et al.*, 2009; Hoffmann *et al.*, 2009; Kaufmann *et al.*, 2009, 2012; Kiel *et al.*, 2009; Nielsen *et al.*, 2010; Sanders *et al.*, 2011; Silbermayr *et al.*, 2011; Ander *et al.*, 2012; Purse *et al.*, 2012) and emphasize the potentially high vector capacity of these species. Consequently, once introduced, arboviruses for which these species have vectorial competence will spread rapidly over large regions of Europe when hosts and appropriate environmental conditions are present. Interestingly, these species have been previously identified as putative vectors of BTV (De Liberato *et al.*, 2005; Savini *et al.*, 2005; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007; Dijkstra *et al.*, 2008; Carpenter *et al.*, 2008a; Vanbinst *et al.*, 2009) and more recently as vectors of SBV (except *C. pulicaris*) (De Regge *et al.*, 2012; Rasmussen *et al.*, 2012; Goffredo *et al.*, 2013; Elbers *et al.*, 2013a, 2013b; Larska *et al.*, 2013a, 2013b), two viruses that have spread across a large part of Europe in a relatively short period.

The monitoring results show that many other species are present, but at lower relative abundances and sometimes only for limited periods of the year. This suggests that these species are less likely to be capable of spreading a vector-borne disease over a large area if they represent the sole competent vector present. Just as our knowledge regarding the biology (e.g. host preferences, breeding sites, activity patterns, etc.) of these less abundant species is rather limited, we also know little about their vector competence because all studies on arbovirus detection in field-collected midges focus strongly on the abundant species. Future studies are therefore necessary to address these aspects.

Factors such as climate, temperature, altitude, soil type and land cover are described as influencing the abundance and distribution of midges (Conte *et al.*, 2007; Mehlhorn *et al.*, 2009; Kirkeby *et al.*, 2010; Pérez *et al.*, 2012). In general, these seem to be applicable to observations recorded during the monitoring in Belgium. Belgium has a temperate maritime climate influenced by the North Sea and Atlantic Ocean, with cool summers and moderate winters (Cfb climate, Köppen–Geiger classification). The relatively high numbers of *Culicoides* collected with OVI traps in 2011 compared with previous years (data not shown) can probably be explained by the high average temperatures of 2011, which was the hottest year ever recorded in Belgium (www.meteo.be/meteo/view/nl/7609555-2011.html). The peak in the number of *Culicoides* collected at week 19 at all sites followed a period of exceptionally high day and night temperatures at the end of April (www.meteo.be/meteo/view/nl/5688544-April+2011.html). In the central and southern parts of the country, midge activity starts about 2–3 weeks later than in the east and northeast regions. This reflects the higher altitudes and associated lower temperatures in these places. Further, the sandy soil type of the northeast favours the earlier maturation of midge larvae because these soils warm up more easily during sunny days in early spring. The differences observed in the probability of collecting high numbers of *Culicoides* at different locations in the region of Liège may derive from the fact that the locations at which monitoring was carried out do not all belong within the same ecoregion. Bettincourt and Verlaine are situated in the loam region, which is characterized as an open agricultural region, whereas Boncelles,

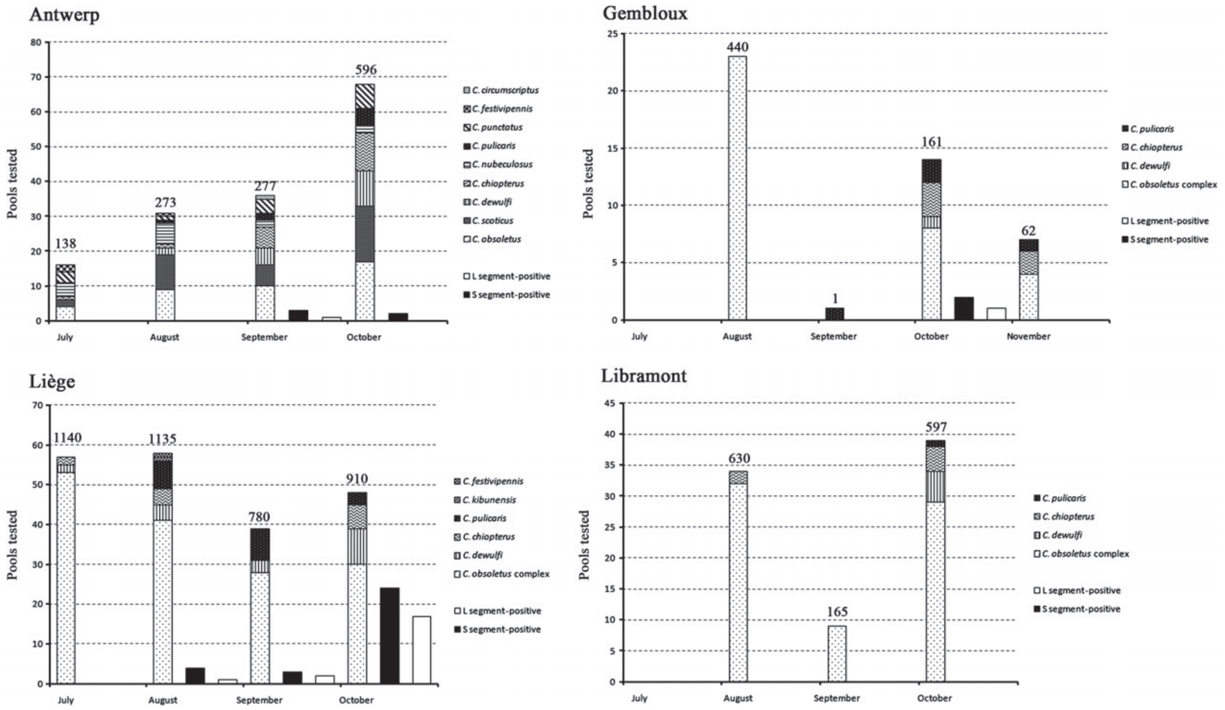

Fig. 3. Findings in pools of *Culicoides* heads originating from four regions in Belgium in 2011 tested for the presence of Schmallenberg virus (SBV) by real-time reverse-transcription polymerase chain reaction. Numbers above the bars indicate the exact numbers of *Culicoides* tested.

Sart Tilman and Nandrin are located in the Condroz. The latter consists of a patchwork of prairies and mostly deciduous forests (National Committee of Geography, 2012). These forests may provide suitable breeding sites for *Culicoides*, especially for the highly abundant *C. obsoletus* complex species, which are known to prefer forest habitats and shaded breeding sites (Kettle, 1962; Conte *et al.*, 2007). Ecological parameters also have important impact on species diversity. For example, species distribution in the bogs of a Belgian nature reserve (Zimmer *et al.*, 2013) was completely different from that found at trapping locations where livestock were present. Proximity to livestock has also been reported as an important factor influencing the number of *Culicoides* collected (Kaufmann *et al.*, 2009; Tschuor *et al.*, 2009; Aybar *et al.*, 2010; Venter *et al.*, 2011). However, in the present study, no clear correlation was found between the presence of horses or other (combinations of) hosts at collection locations and the probability of collecting large numbers of *Culicoides* with OVI traps.

The high variability in species abundance, seasonality and species diversity at individual, even nearby, locations is an important finding of this study because it shows that the outcome of vector monitoring programmes can be substantially influenced by the selection of trapping locations. Previous authors have reported that abundance data obtained with one light trap should not be generalized to a large area (Kirkeby *et al.*, 2013). In order to analyse whether the variation observed between sites is consistent over several years, it would be interesting to obtain monitoring data at fixed locations for several vector seasons. Interestingly, the variation observed at individual collection sites was lost when parameters were analysed at regional level.

The selection of collection sites becomes even more important when monitoring results are used for decision making. One of the most relevant reasons for installing *Culicoides* monitoring in several European countries after the BTV-8 outbreak of 2006 was to define a vector-free period. Our results show that in Belgium in 2011, this period was limited to January. Although regional differences were observed, it is impossible to take these into account in national regulations. To accommodate local variation or the accidental presence of only a limited number of *Culicoides*, a minimal presence of five parous *Culicoides* in a trap as described in the EC legislation 2007/1266 was used as a criterion for offical declariation of vector free periods. As this legislation mentions, in addition to the requirement for a sufficient number of vectors to allow virus transmission, environmental temperature should also be taken into account because this factor influences whether or not virus replication will take place in the vector (Mellor *et al.*, 2000; Carpenter *et al.*, 2011). The observed variation between individual trapping locations in this study and the associated potential influence on decision making suggests the need to define criteria for trap types and trapping locations that can be used for legislative purposes, and indicates that current data on vector-free periods should be interpreted with care.

Earlier reported differences between trap types (Fassotte *et al.*, 2008; Carpenter *et al.*, 2008b; Gerry *et al.*, 2009; Venter *et al.*, 2009) are also reflected in our results. Collections obtained by OVI traps at animal level are generally more abundant than those of RSTs obtained at a height of 12 m. However, the use of ultraviolet (UV) light as an attractant in OVI traps can bias the relative abundances of several species. For example,

Table 6. Detailed overview of pools of *Culicoides* heads found to be positive for Schmallenberg virus by real-time reverse-transcription polymerase chain reaction.

			Ct value		
Location	*Culicoides* species	Collection date	IC	L segment	S segment
Antwerp					
Betekom	*C. obsoletus*	7 Sept 2011	31.7	37.00	34.90
Eindhout	*C. obsoletus*	20 Sept 2011	16.3	Negative	36.45
Berlaar	*C. obsoletus*	6 Sept 2011/20 Sept 2011	17.6	Negative	36.31
Betekom	*C. pulicaris*	7 Sept 2011/4 Oct 2011	14.3	Negative	37.90
Betekom	*C. dewulfi*	4 Oct 2011	16.7	Negative	38.10
Liège					
Boncelles	*C. obsoletus* complex	10 Aug 2011	15.09	Negative	35.41
Sart Tilman	*C. obsoletus* complex	23 Aug 2011	18.94	Negative	35.25
Bettincourt	*C. obsoletus* complex	23 Aug 2011	18.09	Negative	35.96
Bettincourt	*C. dewulfi*	23 Aug 2011	17.11	34.12	32.21
Boncelles	*C. obsoletus* complex	25 Sept 2011	17.96	32.81	30.70
Nandrin	*C. obsoletus* complex	25 Sept 2011	17.05	Negative	33.47
Nandrin	*C. obsoletus* complex	25 Sept 2011	17.59	33.48	32.32
Verlaine	*C. obsoletus* complex	5 Oct 2011	24.82	32.28	31.49
Boncelles	*C. obsoletus* complex	5 Oct 2011	26.78	33.66	34.78
Boncelles	*C. obsoletus* complex	5 Oct 2011	26.67	33.67	32.51
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	31.92	Negative	37.79
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	30.53	34.12	37.49
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	27.24	Negative	38.20
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	29.12	32.05	30.94
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	30.01	Negative	38.31
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	30.75	35.08	36.22
Sart Tilman	*C. obsoletus* complex	5 Oct 2011	29.22	Negative	38.52
Sart Tilman	*C. dewulfi*	5 Oct 2011	24.69	Negative	38.36
Sart Tilman	*C. dewulfi*	5 Oct 2011	29.02	31.97	30.69
Sart Tilman	*C. dewulfi*	5 Oct 2011	26.63	Negative	38.10
Sart Tilman	*C. dewulfi*	5 Oct 2011	29.68	Negative	38.33
Nandrin	*C. obsoletus* complex	14 Oct 2011	21.63	29.80	28.07
Verlaine	*C. obsoletus* complex	28 Oct 2011	26.96	30.56	30.05
Verlaine	*C. obsoletus* complex	28 Oct 2011	20.95	27.95	27.87
Verlaine	*C. obsoletus* complex	28 Oct 2011	26.09	30.36	30.56
Nandrin	*C. obsoletus* complex	28 Oct 2011	24.02	29.66	28.62
Nandrin	*C. obsoletus* complex	28 Oct 2011	24.58	28.60	27.48
Nandrin	*C. obsoletus* complex	28 Oct 2011	24.28	31.39	30.81
Nandrin	*C. obsoletus* complex	28 Oct 2011	24.62	32.80	33.16
Nandrin	*C. obsoletus* complex	28 Oct 2011	23.47	33.37	35.17
Bettincourt	*C. chiopterus*	28 Oct 2011	25.76	29.49	28.69
Gembloux	*C. obsoletus* complex	4 Oct 2011	16.06	Negative	35.35
Gembloux	*C. obsoletus* complex∗	11 Oct 2011	16.22	30.65	26.65

∗Blood and eggs.
IC, internal control.

the relative abundances of *C. pulicaris* and *C. chiopterus* are probably underestimated with OVI traps because these species are, respectively, active during the afternoon before sunset (Goetghebuer, 1919) or are less attracted by UV light (Carpenter *et al.*, 2008b). This is confirmed by the higher relative abundances of both these species in collections obtained with RSTs, which passively collect *Culicoides* throughout the day at a height of 12 m. Despite the fact that RSTs collect midges from a different niche of the *Culicoides* population and that only low numbers of midges were collected in these traps compared with OVI traps, 29 different species were identified, including *C. riouxi*, which was not collected by OVI traps.

The midge collections obtained during 2011 also represent ideal material for the study of several aspects of the epidemiology of SBV. Data on the rRT-PCR detection of SBV in a limited number of pools (representing 1976 midges) from Antwerp and Liège originating from the 2011 monitoring in Belgium have been published previously (De Regge *et al.*, 2012) and indicate that SBV was detected in the heads of *C. obsoletus* complex, *C. dewulfi* and *C. chiopterus* midges. In the present study, an extra 5400 *Culicoides*, some of which originated from an additional six locations, were tested, but no additional putative vector species were found. Only one of the 31 pools of *C. pulicaris* tested was positive for the S segment of SBV. However, as this

Table 7. Numbers and percentages of pools positive for Schmallenberg virus S segment RNA by location in the region of Liège collected during August–October 2011.

	Pools tested, *n*	Pools negative, *n*	Pools positive, *n*	Positivity, %
Verlaine	27	23	4	15
Boncelles	28	24	4	14
Sart Tilman	41	29	12	29
Nandrin	33	25	8	24
Bettincourt	16	13	3	19

pool was negative for the L segment, the role of this species in the spread of SBV remains questionable.

As discussed previously (De Regge *et al.*, 2012), the fact that the pools examined consisted exclusively of heads is an additional indication that the species found to be positive for the S and L segments can act as real amplification vectors because the virus has reached the salivary glands. The Ct values obtained in the heads are in line with Ct values found in SBV-infected *C. sonorensis* midges that have been shown to be orally susceptible to SBV (Veronesi *et al.*, 2013b). Further research will be required to show whether the large range of Ct values obtained (between 26.6 and 38.5 for the S segment) reflect differences in the capacity of the virus to replicate in the salivary glands of individual midges or whether this simply reflects the collection of midges at different periods after the bloodmeal.

Published results have already shown findings of SBV-positive midges in Antwerp and in the Liège region, with a high number of SBV-positive pools recorded in the latter region in October (De Regge *et al.*, 2012). This was confirmed by the testing of additional pools originating from other locations in the same region. More than 50% of pools containing *C. obsoletus* complex midges were found to be SBV-positive, indicating a minimum infection prevalence of 3.1% (19/620) in this species in October at Liège, given each positive pool contained one SBV-positive midge. A similar infection prevalence of 3.6% (3/83) was found in *C. obsoletus s.s.* midges in September in Antwerp. Despite the higher probability for obtaining larger *Culicoides* collections at Boncelles, Sart Tilman and Nandrin than at Verlaine and Bettincourt, no significant differences in the number of SBV-positive pools were found between the different sites, indicating that SBV-infected midges were evenly distributed across the Liège region. The results of this study also show that SBV-positive *Culicoides* were found at Gembloux in the centre of Belgium. However, at Libramont in the south of the country, none of the pools tested positive for SBV. This is probably indicative of a lesser spread of the virus to this part of Belgium, which is characterized by higher altitudes and dense forests. It also correlates with the relatively lower within-herd seroprevalence found in sheep and cows in this region at the end of the vector season of 2011 (Méroc *et al.*, 2013, 2014). The finding of high numbers of SBV-positive midges at Libramont during the vector season of 2012, when the prevalence of infection in *Avaritia* midges was found to be 2.86% in August (De Regge *et al.*, 2014), supports this hypothesis.

The rRT-PCR screening of *Culicoides* for the presence of SBV does not provide any indication of the circulation of SBV in Belgium prior to August 2011. However, this remains only indicative because a low level of circulation of the virus before August may easily have been missed by this approach. Nevertheless, SBV circulation from August coincides with the period in which regional animal health care centres [Association Régionale de Santé et d'Identification Animales (ARSIA) and Dierengezondheidszorg Vlaanderen (DGZ)] in Belgium began to receive notifications of problems such as milk drop and diarrhoea on cattle farms from which other endemic and epizootic viruses (e.g. bovine viral diarrhoea virus, infectious bovine rhinotracheitis virus, BTV) had been excluded.

The results of the present study underline the variation associated with the collection locations selected and the trap types used, but also show the advantages of a *Culicoides* monitoring programme. However, the financial aspects of such a scheme make it difficult to justify such extensive yearly monitoring, certainly as Belgium became officially free of BTV in February 2012. Nonetheless, as the odds are high that (re)new(ed) introductions of *Culicoides*-borne or other vector-borne diseases will occur in the future, it would seem advisable to install a permanent and structural surveillance system, whether or not this functions in rotation with the monitoring of other vectors such as mosquitoes and ticks. This is necessary not only to retain in place expertise related to vector trapping and identification, but particularly to detect the introduction of exotic pathogens and vectors as early as possible and to correctly assess any potential risks to animal and public health associated with potential introductions of arthropod-borne disease.

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