

Host-Seeking Activity of Bluetongue Virus Vectors: Endo/Exophagy and Circadian Rhythm of *Culicoides* in Western Europe

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Abstract

Feeding success of free-living hematophagous insects depends on their ability to be active when hosts are available and to reach places where hosts are accessible. When the hematophagous insect is a vector of pathogens, determining the components of host-seeking behavior is of primary interest for the assessment of transmission risk. Our aim was to describe endo/exophagy and circadian host-seeking activity of Palaearctic *Culicoides* species, which are major biting pests and arbovirus vectors, using drop traps and suction traps baited with four sheep, as bluetongue virus hosts. Collections were carried out in the field, a largely-open stable and an enclosed stable during six collection periods of 24 hours in April/May, in late June and in September/October 2010 in western France. A total of 986 *Culicoides* belonging to 13 species, mainly *C. brunnicans* and *C. obsoletus*, was collected on animal baits. *Culicoides brunnicans* was clearly exophagic, whereas *C. obsoletus* was able to enter stables. *Culicoides brunnicans* exhibited a bimodal pattern of host-seeking activity with peaks just after sunrise and sunset. *Culicoides obsoletus* was active before sunset in spring and autumn and after sunset in summer, thus illustrating influence of other parameters than light, especially temperature. Description of host-seeking behaviors allowed us to discuss control strategies for transmission of *Culicoides*-borne pathogens, such as bluetongue virus. However, practical vector-control recommendations are difficult to provide because of the variation in the degree of endophagy and time of host-seeking activity.

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Introduction

Host-parasite systems are subject to opposing selective processes: on the one hand, parasite fitness is increased by a higher frequency of encounters with hosts and on the other hand, host fitness is increased by avoiding these contacts [1]. Feeding success of free-living hematophagous insects, which can be considered parasites of vertebrates with periodic and brief contacts, depends on their ability to be active when hosts are available and to reach places where hosts are accessible [2]. Circadian and seasonal activities of insect are regulated by endogenous oscillators which are initiated by natural diel alternation of light and darkness [3]. These biological clocks enable organisms to anticipate variations of biotic and abiotic factors associated with seasonal or diel progression rather than follow them [3,4]. Diel timing of host-seeking activity should be selected to ensure temporal encounter of the insect and host and to minimize the risk of dying during host-seeking activities. Similarly, endophagy, defined as the trend of

obtaining a blood meal within a man-made structure [3], can be regarded as an adaptation of hematophagous insect to reach their hosts.

The description of circadian activity and endo/exophagy of hematophagous insects could have practical implications for the control of diseases induced by transmitted pathogens. A well-known example is stabling horses at night in South Africa where *Culicoides imicola* Kieffer is known to be nocturnal and mainly exophagic [5,6]. This vector-control strategy has proven to be an environmentally-friendly and inexpensive method to prevent African horse sickness (AHS) by reducing bites of the main vector. From 2006 to 2008, Europe faced a huge epizootic of bluetongue virus serotype 8 (BTV8) transmission, leading to disastrous sanitary consequences in domestic ruminant populations and to important disruptions in animal trade [7]. European regulations recommend the stabling of animals during *Culicoides* activity to reduce BTV transmission (EU Council

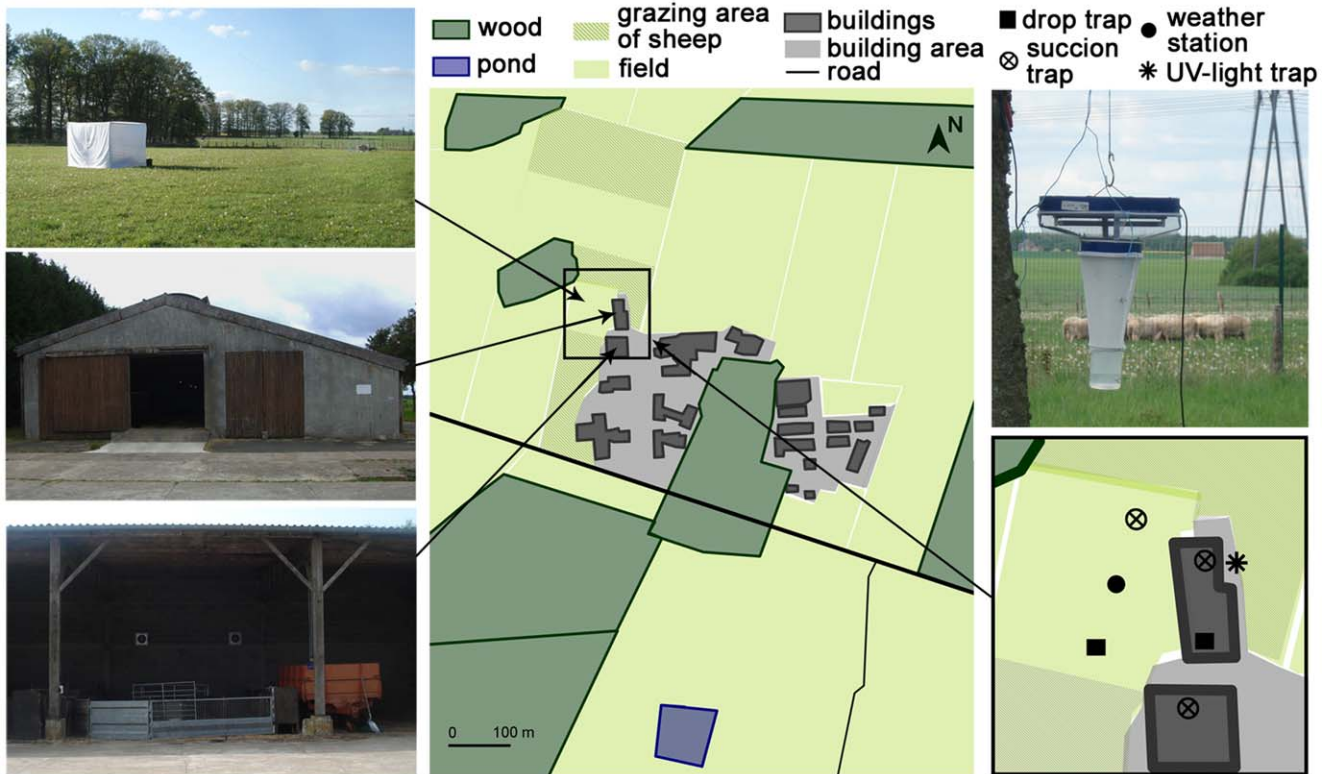


Figure 1. Climatic conditions in the study site. A. Ombrothermic diagram for comparison between 2010 and the 1971–2000 period (data from Météo-France station of Parçay-Meslay); B. Boxplots of temperature (°C) and relative humidity (%) recorded by data loggers at each location during the 15 collection sessions; C. Boxplots of mean and maximum wind speed (km/h) recorded by the local weather station during each of the 6 summer collections.

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Directive 2000/75/EC). The direct costs due to indoor housing of animals in The Netherlands in 2006, which was compulsory in restriction zones, were estimated to 18 million Euros, *i.e.* 55% of total economic impact [8]. However, endo/exophagy of Palearctic *Culicoides* was not precisely described and then benefits of this housing strategy were uncertain. The recent emergence of a novel Orthobunyavirus, named Schmallenberg virus [9], in European ruminant populations highlighted the crucial need of address this issue.

Endophagous behavior of Palearctic species was reported first by Overgaard Nielsen and Christensen [10] in Denmark. In late 2006, comparing UV-light trap collections inside and outside sheds in The Netherlands, Meiswinkel *et al.* [11] collected threefold more *Culicoides* outside than inside, where cattle were kept for the night. The species collected mainly inside, *i.e.* *Culicoides obsoletus* Meigen/*Culicoides scoticus* Downes and Kettle, *Culicoides dewulfi* Goetghebuer and *Culicoides chiopterus* (Meigen), were suspected to be involved in BTV8 transmission [11]. Using the same method in France at the same period, Baldet *et al.* [12] collected mainly the same species inside stables, but in greater number inside than outside. Authors conceded that the results were difficult to compare and interpret because of the variation in building openings and in cattle abundance close to the trap in the different collection sites [12]. With a standardized approach, Baylis *et al.* [13] highlighted that the presence of animals and the opening of stable increased the indoor number of *Culicoides* collected by UV-light trap. Moreover, authors showed that the decrease of *Culicoides* number between outside and inside traps is greater in summer

(6.5 fold) than in autumn (3 fold) [13]. Unfortunately, all these studies used UV-light traps to assess *Culicoides* abundance, which do not assess correctly the biting rate on animals [14–16]. Indeed, light attraction might lead to an overestimation of the *Culicoides* endophagy, due to a higher trapping efficiency when light traps are set inside stables, rather than outside [17].

Similarly, circadian cycles of Palearctic species are poorly described. It is widely assumed that *Culicoides* are mostly crepuscular and may continue to be active throughout the night. However, many species are also troublesome in the day displaying two biting peaks: one after sunrise and the other close to sunset. Hours of midge attacks can lengthen when low-light overcast conditions prevail, leading to biting throughout the day in both open (*Culicoides impunctatus* Goetghebuer) and forested environments (*C. obsoletus*) [17]. However, only a few studies have described rigorously *Culicoides* nycthemeral cycles with consecutive 24 h collections [18,19], and only one has utilized host-seeking behavior of European *Culicoides* [20]. Using horse-baited collections, the latter found that the largest number of *C. obsoletus* was collected at sunset, far less at sunrise and occasionally in the afternoon and night.

The aim of this study was to describe circadian host-seeking activity and endo/exophagous behavior of Palearctic *Culicoides* using host-baited trap collections. As insect behavior is directly affected by geophysical (sun and moon light cycles) and climatic (temperature, humidity, wind) factors, we carried out collections in three different periods of the year.

Results

Climatic Data

Climate at the study site was oceanic, with a mean annual temperature of 11.4°C, thermal amplitude of 14.9°C and annual rainfall of 694 mm (Météo-France data, 1971–2000). In 2010, annual rainfall was lower than for the 1971–2000 period (586 mm), with deficit in rainfall during the first part of the year (179 mm between January and May 2010 versus 298 mm for the reference period). Temperatures were generally close to normal values in 2010, with colder values in January and December and warmer in June and July (Fig. 1A).

During collection sessions, temperatures were higher in summer than in autumn and spring, with smaller amplitude in autumn, whereas humidity was higher in autumn than in summer and spring (Fig. 1B). The temperature decreased gradually from indoors to outdoors, while humidity showed the inverse tendency. In autumn, however, the relative humidity was the highest in the largely-open stable (Fig. 1B). Amplitudes of temperature and humidity were higher outdoors than in other locations, with outlier observations, especially in autumn, probably due to direct exposure of recording devices to the sun. Wind speeds were correlated between locations, with an expected wind gradient from outdoors to indoors, with an intermediate situation for the largely-open stable (Fig. 1C).

Diversity and Seasonality

During the 18 collection sessions, a total of 986 *Culicoides* (948 females and 38 males) belonging to 13 species was collected in host-baited traps (drop trap and suction trap) and 539 *Culicoides* (451 females and 88 males) belonging to 19 species in the UV-light/suction trap (Table 1). Molecular assays were performed on 407 *Culicoides* females morphologically identified as belonging to the Obsoletus Complex. The sample contained 349 *C. obsoletus*, 25 *C. scoticus*, 6 *C. dewulfi* and 1 *C. chiopterus*; 26 individuals were not identified. Only 4% of *Culicoides* collected in host-baited traps were males – mainly the dominant species *Culicoides brunnicans* Edwards collected by the drop trap – whereas 20% of *Culicoides* were males in UV-light/suction trap. A total of 84 gravid females was collected: all in the suction trap mainly in summer (47%) and most were *C. obsoletus* (76%). Blood-fed females were collected by all traps, but mainly by the drop trap (197 versus 4 in suction trap and 9 in the UV-light/suction trap). The majority of blood-fed females (196/210) belonged to the dominant species *C. brunnicans* and *C. obsoletus*; the engorgement rate in the drop trap was 28% for *C. brunnicans* and 65% for *C. obsoletus*.

Diversity and abundance of species varied across seasons and traps. *Culicoides brunnicans* was present almost exclusively in spring (Table 1). *Culicoides obsoletus* was present during the three periods, mainly in summer (Table 1). The abundance of *C. scoticus* decreased progressively from the first to the last collection period (Table 1). *Culicoides brunnicans* was more abundant in host-baited traps than in the UV-light/suction trap, whereas the opposite situation was observed for *C. obsoletus*. Moreover, the UV-light trap collected some species rarely or not collected in host-baited traps, as *Culicoides punctatus* Latreille and *Culicoides festivipennis* Keiffer. On the contrary *C. dewulfi* and *C. chiopterus* were found in host-baited traps but not in the UV-light/suction trap (Table 1).

Endo/exophagy

The best model predicting the observed abundance of *Culicoides* overall (Pearson's product-moment correlation, $r=0.98$) was the complete model including location, season, interactions between both and trap as fixed effects and the session as a random effect,

even though season lacked a clear effect on abundance (Table 2). Most *Culicoides* females were collected outdoors (mean predicted number of *Culicoides* was 8.5 outdoors versus between 0.4 and 1.0 indoors) and more were collected by the drop trap (4.2 versus 2.4).

The best model predicting *C. brunnicans* abundance ($r=0.99$) was the complete model without the interaction term (Table 2). This species was found almost exclusively during the first collection period and outdoors (between 100 and 300 fold more abundant outdoors than indoors) and mainly by the drop trap (1.9 versus 0.5 by the suction trap). Interaction between location and season ($p<0.001$) was needed to obtain the best prediction of *C. obsoletus* abundance ($r=0.91$). *Culicoides obsoletus* was collected more abundantly outdoors (between 8 and 10 fold more abundant outdoors than indoors) and by the suction trap (1.6 versus 0.9 by the drop trap). For *C. scoticus* and *C. dewulfi*, only trap and location as fixed effect were needed to obtain parsimonious models with good fit ($r=0.98$ and $r=0.72$). Both species were collected more abundantly by the suction trap (predicted abundance was 0.22 and 0.26 versus 0.04 and 0.04), and *C. scoticus* was collected more abundantly outdoors (between 9 and 22 fold more abundant outdoor than indoor).

Circadian Host-seeking Activity

During collections, the time of sunrise ranged from 6h49 (GMT +1) the 26th April to 8h06 the 8th October, when the sunset ranged from 21h01 to 19h24 between the same dates.

In spring, *C. brunnicans* exhibited two peaks of diel activity. Host-seeking activity increased rapidly just after sunrise, and then slowly decreased during the next 5 hours. Host-seeking activity started again 5 hours before the sunset, reaching a peak just after sunset and then decreasing rapidly (Fig. 2).

Host-seeking activity of *C. obsoletus* was recorded mainly around sunset. In spring and autumn, host-seeking activity started slowly 2 hours before sunset, peaked just before sunset and then decreased rapidly (Fig. 2). In summer, we observed a similar pattern of host-seeking activity except that the peak occurred just after sunset. Minor host-seeking activity was recorded around sunrise, especially in autumn. Outside these 2 periods, host-seeking activity ceased. The surprising peak observed in the middle of the afternoon (15h20) was the result of a single collection with the drop trap.

The same pattern, *i.e.* host-seeking activity mainly around sunset and a small amount around sunrise, was observed for *C. dewulfi*, *C. chiopterus* and *C. scoticus*, even if the low number of females collected did not provide details of the daily host-seeking activity for these 3 species.

Almost all the collections were carried out without any rainfall. Comparing *Culicoides* abundance with data logger records, we noted that *Culicoides* were collected across a large range of temperature (Fig. 3): single individuals were collected at temperatures as lowest as 4.8°C and the first substantial catch (60 *Culicoides*) with low temperatures was recorded at temperatures between 8.4 and 10.4°C. Low relative humidity did not seem to inhibit host-seeking activity (Fig. 3) as 47 *Culicoides* were collected between 32 and 52% relative humidity. Moreover, we noted that the first significant catch (16 *Culicoides*) with strong wind was recorded with a wind speed of 5 m/s, but only 2/3 of collection sessions could be analyzed. Indeed, due to technical problems, local meteorological parameters were missing for some collection sessions.

Culicoides brunnicans biting rates in spring assessed by drop trap or suction trap were linearly and positively correlated with the abundance in the UV-light/suction trap ($R^2=0.55$, $p<0.001$), but some collections were positive in host-baited traps but not in UV-

Table 1. Numbers of *Culicoides* collected over 18 sessions in spring, summer and autumn by host-baited traps and UV-light/suction trap.

Location	R ¹	Species	Host-baited traps												UV-light/suction trap											
			Suction Trap						Drop Trap						Total						Total					
			F ²	M	F	P	F	P	M	F	P	M	F	P	F	M	F	P	M	F	P	M	F	P		
OUTDOORS	Field	1	<i>C. brunnicans</i>	592	24	111	11	475	50	24	6	67	140	62	126	5	62	14	93							
		2	<i>C. obsoletus</i>	193	3	31	55	61	54	27	63	2	17	65	42	60	1	15	40	186	16	17	59			
		3	<i>C. scoticus</i>	43		29	81	5	40	2			6	83			1		29	2	18	33	8			
		4	<i>C. punctatus</i>	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	27	4	7	57	20			
		5	<i>C. festivipennis</i>																22				11			
		6	<i>C. pulicaris</i>	8	2	1	1	5	40				12	1					12	1			12			
		7	<i>C. dewulfi</i>	16	5	3	3	6	33	1	1		4						4				4			
		8	<i>C. chiopterus</i>	10	4	5	100												6	6			6			
		9	<i>C. poperinghensis</i>	2				2																		
		10	<i>C. achirayi</i>																4				4			
		10	<i>C. lupicaris</i>	1		1							3	2					3	2			1			
		10	<i>C. vexans</i>	3	2			1					1	1					1	1			1			
		13	<i>C. nubeculosus</i>	3				1				1														
		13	<i>C. simulator</i>										3						3				3			
		15	<i>C. kibunensis</i>										2						2				2			
		15	<i>C. santonicus</i>	1	1								1	2	1	2			1	2	1	2				
		17	<i>C. fascipennis</i>										1						1				1			
		17	<i>C. pictipennis</i>										1						1				1			
		17	<i>C. salinarius</i>										1						1				1			
		17	<i>C. puncticallis</i>										1						1				1			
			Obsoletus Complex	24	3	9	67	2				9	55	1					10	1			6			
			Pulicaris Group	1				1											1				6			
			Total Field	900	29	189	86	38	4	514	24	55	1	18	451	88	174	64	164	4	113	4	113			
INDOORS	Largely open stable	1	<i>C. brunnicans</i>	1	1								38	4					1				1			
		2	<i>C. obsoletus</i>	13	2	1	3	9	56	2																
		3	<i>C. scoticus</i>	4	2			2																		
		7	<i>C. dewulfi</i>	1				1																		
		17	<i>C. newsteadii</i>	1		1		1																		
			Obsoletus Complex	1				1																		
			Total Largely open stable	21	2	4	5	12	2	-	-	-	-	12	2	-	-	-	-	-	-	-	-			
	Closed stable	1	<i>C. brunnicans</i>	2	1			1																		
		2	<i>C. obsoletus</i>	18	4	8	25	1	1	7	1	3	1													

Table 1. Cont.

Location	R ¹	Species	Host-baited traps						UV-light/suction trap										
			Suction Trap			Drop Trap			Total			Total							
			Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn					
	3	<i>C. scoticus</i>	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	4	<i>C. punctatus</i>	1																
	8	<i>C. chiopterus</i>	3	1	3														
		Obsoletus Complex	1																
		Total Closed stable	27	7	10	4	2	8	2	58	2	58	6	516	24	56	6	20	
		TOTAL	948	38	203	95	4	2	58	6	516	24	516	64	174	64	164	4	113
																			20

¹R: species rank calculated with the total number of individuals whatever the trap and the season.

²F: females; (P): parity rate as No. parous/No. females (given in percentage if $F > 5$); M: males. For the sake of clarity, 0 were not quoted.

³ND: not done. The drop trap was not used in the largely open stable, and only one UV-light/suction trap was used outdoors.

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light/suction trap, or conversely (Fig. 4). Correlation was much lower for *C. obsoletus* ($R^2 = 0.13$, $p = 0.032$), although it seemed that UV-light/suction trap tended to over-estimate biting rates.

Discussion

We described endo/exophagy and circadian host-seeking activity of Palaearctic *Culicoides* species using drop trap and suction trap collections. The mean number of *Culicoides* per collection was rather low. It illustrated the highly variable *Culicoides* abundance in space and time. Indeed, up to 3,000 *Culicoides* were collected at this site by single UV-light trap night-collections, carried out independently of these collections. The trap, as a fixed effect, was necessary to model abundances of all *Culicoides* species. The drop trap collected more *C. brunnicans* than the suction trap – Viennet *et al.* [16] showed that drop trap collected more *C. brunnicans* than other host-baited trap methods – but abundances assessed by both methods were highly correlated (Pearson's $r = 0.99$). The suction trap collected more individuals of other species than the drop trap, and presence of gravid females suggested that this trap may have collected non host-seeking females. Moreover, differences between traps may reflect spatial variations of *Culicoides* populations. However, satisfactory concordance of abundance ($r = 0.67$) for *C. obsoletus* in both traps suggested that the suction trap could be used as an efficient and easy alternative to drop trap for this species.

To date, investigations of *Culicoides* endo/exophagy had been carried out always with UV-light/suction traps [5,6,11–13,21,22], with the exception of one study [23] on ornithophilic *Culicoides* species. But UV-light/suction trap collections were greatly dependent on local conditions, such as presence and number of animals in the vicinity of trap [24], leading to complicated interpretations. For instance, *C. imicola* was collected in greater numbers by indoor animal-baited light traps than by outdoor unbaited light traps in Spain [21], in apparent contradiction with South-African descriptions, where this species exhibited exophagic behavior [5,6]. In our study, host-baited collections in outdoor and indoor (with two degrees of opening for the host-baited suction trap) conditions highlighted that Palaearctic *Culicoides* are primary exophagous insects. Some species are strictly exophagous, such as *C. brunnicans*, some others show some degree of endophagy, such as *C. obsoletus* or *C. scoticus*. Baylis *et al.* [13] showed that differences between outdoor and indoor catches decreased from summer to autumn. To explain these differences, authors suggested factors difficult to untangle. Colder temperatures and stronger wind in autumn could suppress outdoor *Culicoides* host-seeking activity – overcast conditions have been shown to decrease outdoor UV-light/suction collections [6,11]. On the other hand, autumnal conditions could also interfere in light trap efficiency – wind could modify the trap suction efficiency – or host-seeking activity could occur mainly before sunset late in the year, decreasing part of the *Culicoides* which otherwise would be collected [25,26]. Even though interaction between season and location was necessary to model *C. obsoletus* abundance, our data did not allow to clearly confirm this observation. Finally, most of *Culicoides* females collected indoors in host-baited traps may have been host-seeking females. However, the low number of gravid *C. obsoletus* indoors suggest that some females may enter into sheds to oviposit, as illustrated by the possibility to find *Culicoides* larvae indoors [27,28].

It is widely assumed that *Culicoides* are mostly crepuscular [29]. However, only few studies have described rigorously *Culicoides* circadian cycles with consecutive 24 h collections [18,19,30], and only one has considered host-seeking behavior of European *Culicoides* using horse-baited collections [20]. Two patterns of host-

Table 2. Mean No. observed (max) and predicted *Culicoides* for all species and the most abundant species depending on the trap, the location and the season.

Effect	Value	All species		<i>C. brunnicans</i>		<i>C. obsoletus</i>		<i>C. scoticus</i>		<i>C. dewulfi</i>	
		Observed	Pred ³	Observed	Pred	Observed	Pred	Observed	Pred	Observed	Pred
Trap	Drop trap	16.4(249)	4.18 ^{***}	13.4(237)	1.94 ^{***}	2.1(19)	0.91 ^{***}	0.2(4)	0.04 ^{***}	0.1(1)	0.04 [*]
	Suction trap	6.6(99)	2.37	2.1(63)	0.45	2.7(28)	1.62	0.8(25)	0.22	0.3(3)	0.26
Location	Outdoor	25.0(249)	8.46 ^{***}	16.4(237)	3.55 ^{***}	5.4(28)	3.09 ^{***}	1.2(25)	0.34 ^{***}	0.4(3)	0.41
	LOS ²	1.2(6)	0.99 ^{**}	0.1(1)	0.03	0.7(4)	0.39	0.2(2)	0.04	0.1(1)	0.03
	Indoor	0.8(6)	0.38	0.1(1)	0.01	0.5(5)	0.32	0.1(1)	0.02	0.0	0.00
Season	Spring	24.0(249)	5.62	19.6(237)	3.55	1.9(14)	0.89	1.3(25)	–	0.2(3)	–
	Summer	5.0(34)	2.03	0.2(3)	0.04	3.6(28)	1.53 [°]	0.2(2)	–	0.1(0)	–
	Autumn	2.6(18)	2.17	0.0	0.00	2.0(14)	1.38	0.2(1)	–	0.2(2)	–
Sea*loc ¹	–	p<0.001	–	–	p<0.001	–	–	–	–	–	–

¹Interaction between season and location.

²LOS: largely open stable; Pred: predicted values; NS: not significant.

³Predicted values (Pred) are in bold and underlined if analyze of variance between models with and without that effect showed significant differences with for $\alpha=0.05$. P-values were given for effect modalities compared to a reference value, i.e. "suction trap" for trap, "indoor" for location and "autumn" for season (**p<0.001; *p<0.01; °p<0.05; ° p<0.1, no indication p>0.1).

seeking activity were observed during our 24 consecutive hours of collection. *Culicoides brunnicans* showed a bimodal pattern of host-seeking activity with peaks at dawn and dusk, as found by Blackwell *et al.* [31] for flight activity of *C. impunctatus*. Other species, *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus*, showed a main activity peak around sunset, even if some individuals could be collected around sunrise. We cannot exclude that in context of higher *Culicoides* abundance, these species present a second, but lower peak of host-seeking activity around sunrise, as highlighted by Van der Rijt *et al.* [20] for *C. obsoletus* (84 individuals collected at sunset versus 10 at sunrise). Indeed, Sanders *et al.* [32] observed two peaks of flight activity for *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus* around both sunrise and sunset. Meteorological conditions are known to impact adult midge activity [29,33]. It seems clear that windy conditions suppress the flight activity of *Culicoides* [6,31,33]. Flight activity is described experimentally as exceptional at temperatures below to 10°C and optimal at temperatures above to 20°C for *Culicoides oxystoma* Kieffer and

Culicoides pictipennis (Staeger) [34]. Finally, Blackwell *et al.* [31] found a positive correlation between flight activity of *C. impunctatus* females and relative humidity and rainfall. During our observations, relative humidity was not found to influence host-seeking activity and minimal temperature required seemed in coherence with that described by Tsutsui *et al.* [34]. We found that the highest host-seeking activity occurred in low sunlight, corresponding to twilight periods. Changes in light intensity during the day could explain "abnormal" host-seeking activity. For instance, 15 *C. obsoletus* were collected in the middle of the afternoon (at 15h20) one day of June (Fig. 2). During this afternoon, sunlight decreased from 120 (14h00) to 77 (15h00) and to 20 J/cm² (16h00), compared to means of 226 (14h00), 224 (15h00) and 225 (16h00) the other collection days of June. The first consequence is the possible lengthening of *Culicoides* attack hours when low-light overcast conditions prevail leading to biting throughout the day, as is known in open pastures for *C. impunctatus* or *C. obsoletus* and in forested environments for *C. obsoletus* [17,35]. The other conse-

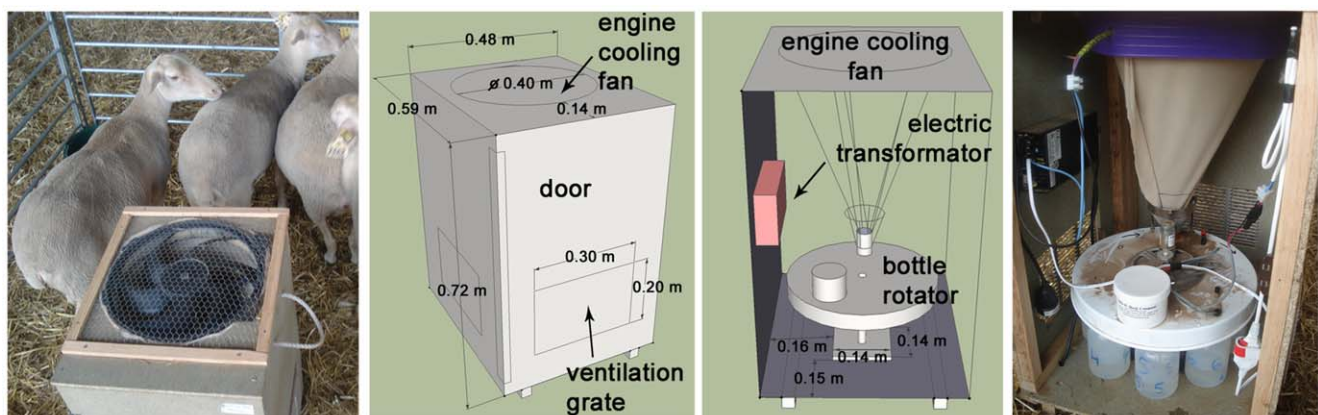


Figure 2. Circadian host-seeking activity of *C. brunnicans* and *C. obsoletus*: total number of females collected outdoor by host-baited traps at each session and day time. Small symbols are single collection and lines with large symbols the means by period. Vertical lines symbolize the time of sunrise and sunset.

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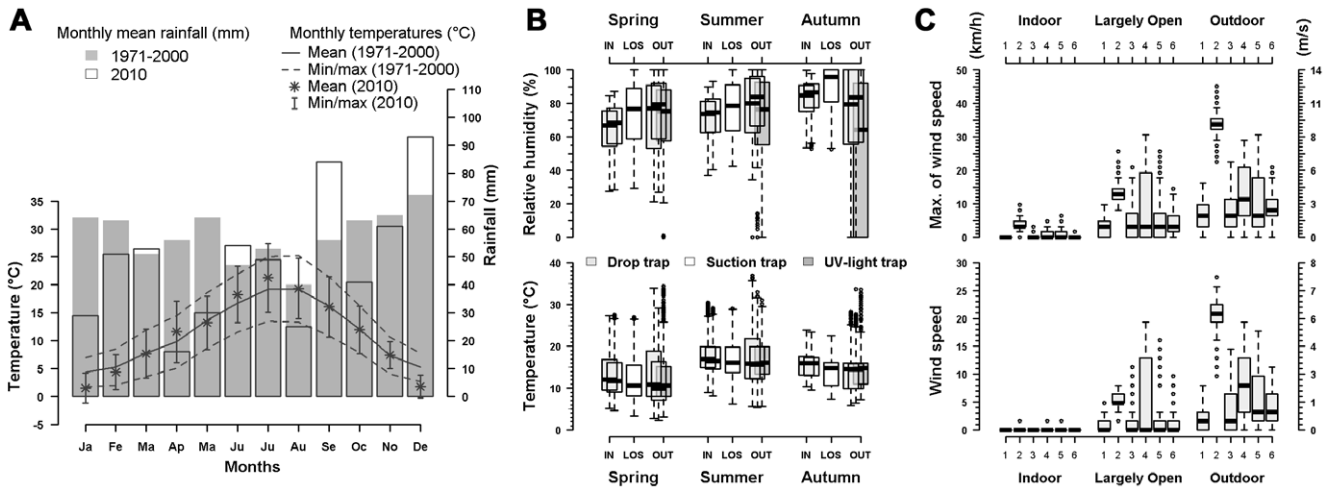


Figure 3. Number of *Culicoides* collected outdoor in host-baited traps compared to temperature and relative humidity recorded by data loggers. Lines are the distribution histogram of meteorological parameters recorded during the collections. doi:10.1371/journal.pone.0048120.g003

quence is the possible nocturnal host-seeking activity of *Culicoides* due to moon illumination, as observed for North-American *Culicoides* species by Barnard and Jones [18] or Lillie *et al.* [19]. Therefore, it would be interesting to study influence of moon light and moon phases on circadian host-seeking activity of Palearctic *Culicoides*. Sunset and sunrise times depend on locality and season, leading to a change in host-seeking activity times throughout the year. Moreover, *C. obsoletus* was active before sunset during spring and autumn, and after sunset during summer, illustrating the influence of other parameters than sunlight. This could be an adaptation to temperatures as suggested by Lillie *et al.* [19] who found the same pattern change throughout the year.

Finally, this work allowed the comparison of biting midge abundance in sheep-baited traps and in UV-light/suction traps,

operated as a standardized method. Due to experimental design, these methods could not be rigorously compared. A positive and high correlation could be established for *C. brunnicans*, showing that UV-light collections may be used without correction to follow changes in biting rates. Correlation was worse for *C. obsoletus*, but the tendency of biting rate over-estimation by the UV-light/suction trap was already recorded by Carpenter *et al.* [14] and Viennet *et al.* [16].

To conclude, in farms with grazing animals, keeping valuable animals in closed stables would limit their risk to be bitten by *Culicoides* and then to be infected by *Culicoides*-borne pathogens, as biting midges seem to be primarily exophagic insects. However, practical recommendations are difficult to provide because of the variation in the degree of endophagy and time of host-seeking

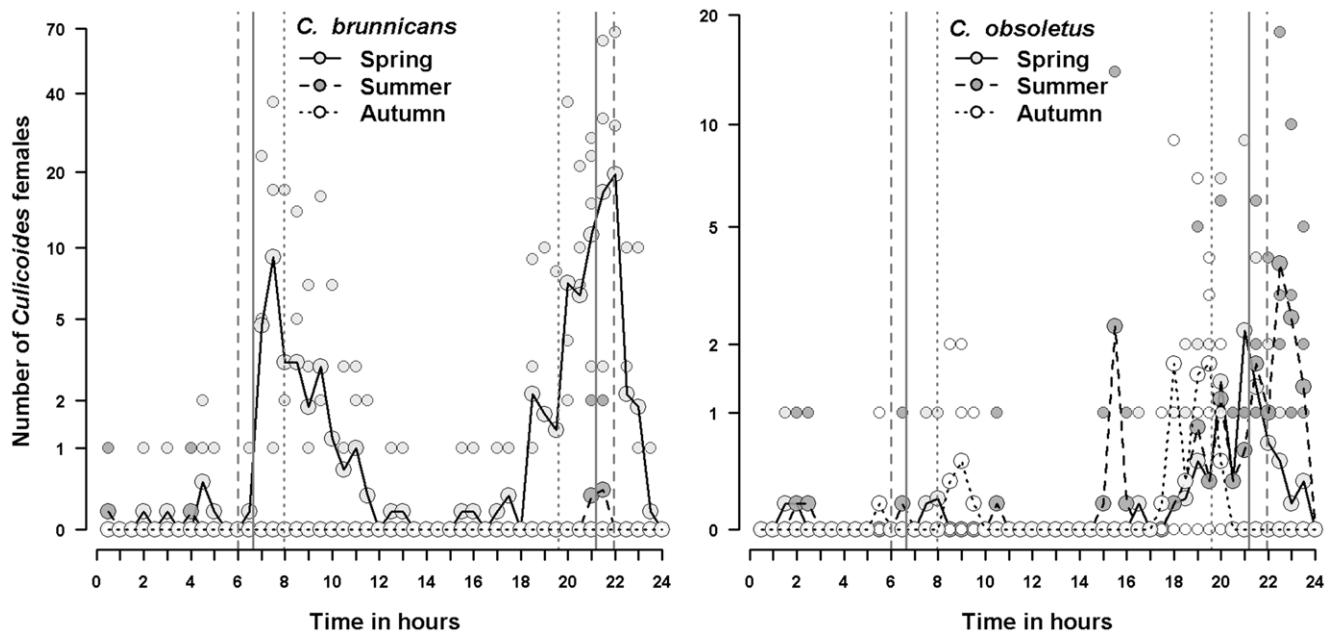


Figure 4. Correlation between the numbers of *C. brunnicans* and *C. obsoletus* collected outdoor in host-baited traps and in UV-light/suction trap. doi:10.1371/journal.pone.0048120.g004

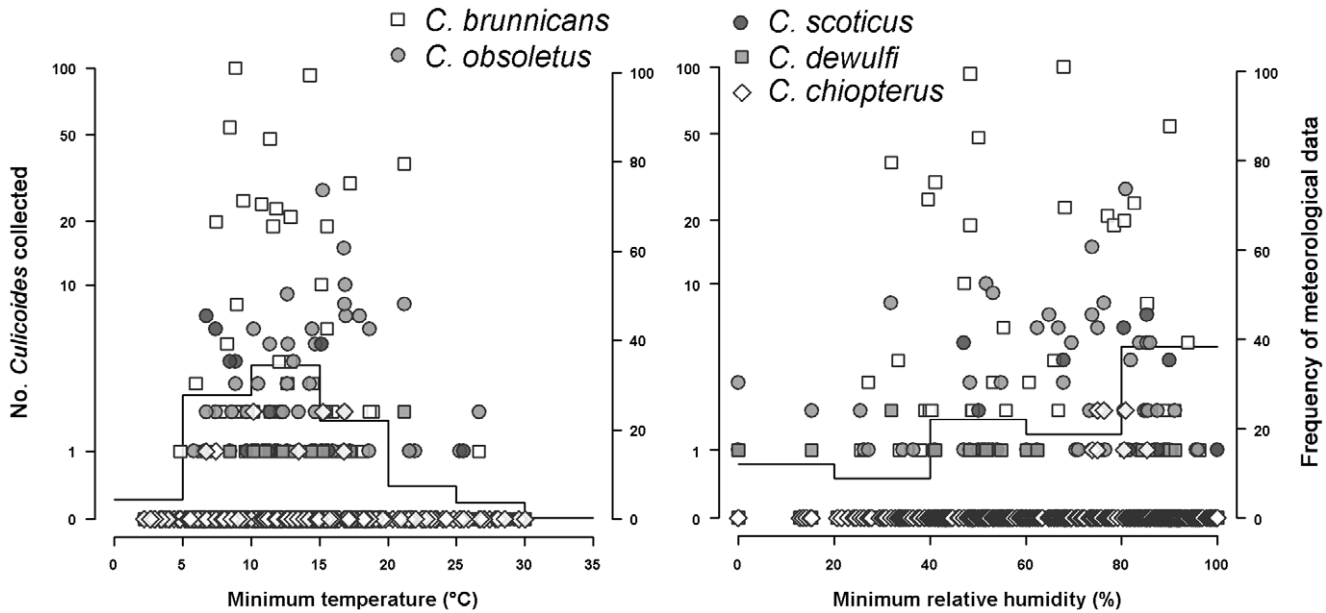


Figure 5. Sketch map of the study site at Nouzilly (western France) with the location of the sheep-baited traps (drop trap and suction trap) in the three sites (field, large-open-stable, closed stable) and of the UV-light/suction trap.
doi:10.1371/journal.pone.0048120.g005

activity. The activity parameters depend on *Culicoides* species, host abundance, season, stabling conditions and weather. This illustrates the consequence of evolutionary processes selecting the ability of hematophagous insects to ensure host encounter in time and space.

Materials and Methods

Study Site and Culicoides Collections

Indoor and outdoor collections were carried out during six collection periods of 24 hours in April/May, in late June and in

September/October 2010 on an experimental farm (Institut National de la Recherche Agronomique, INRA, UE1277 PFIE) breeding sheep and located in Nouzilly (47°33'01''N; 00°47'52''E), western France. Three areas were investigated: a field (61×103 m), a closed stable (17×45 m) and a largely-open stable (30×10 m) (Fig. 5). The closed stable had closed windows (1×0.64 m; 30 windows west side and 12 east side) and two large doors (3.1×3.4 m) half open, thus about 3% of the walls were open surface. The largely open stable was constituted by 3 walls, 1 roof and 1 open side, and openings constituted about 38% of the wall surface. During collections, meteorological conditions (air temper-

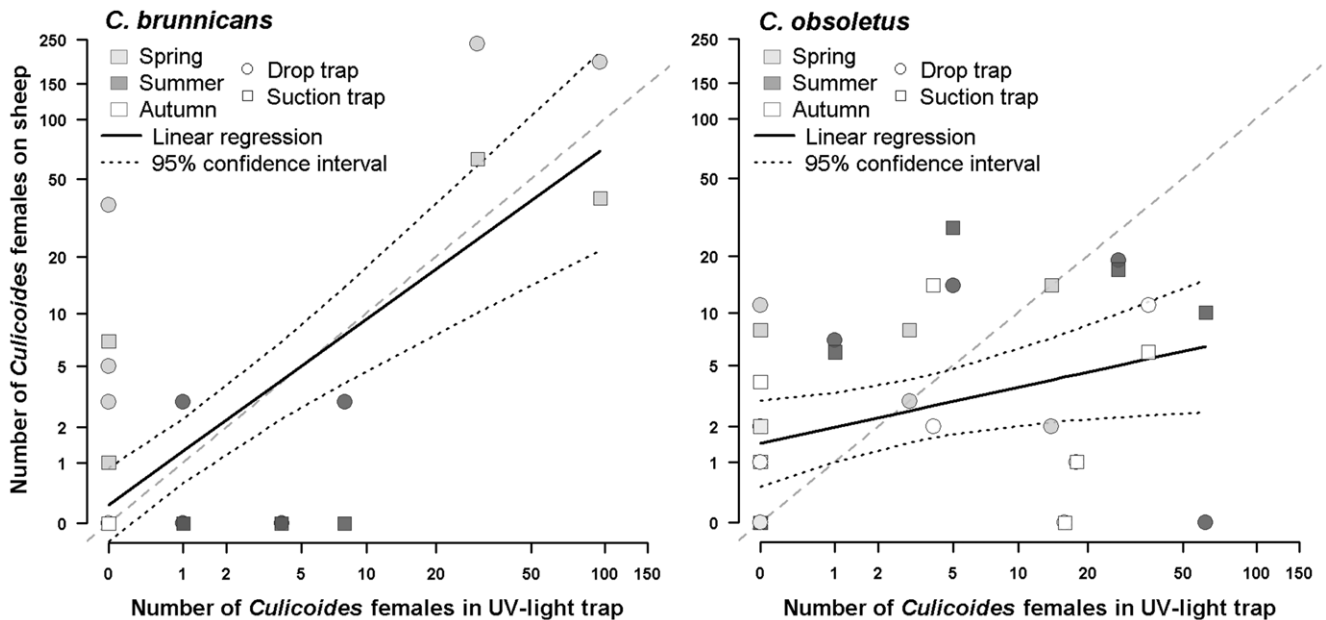


Figure 6. Inside and outside schemes and pictures of the suction trap.
doi:10.1371/journal.pone.0048120.g006

ature, relative humidity, wind speed and direction, solar radiation, rainfall and atmospheric pressure) were recorded every five minutes using a weather station Vantage Pro 2 (Davis Instruments France). Temperature and relative humidity were also recorded by a Tiny Tag TGP-4500 data logger at each trap location. Monthly meteorological data recorded by the national weather station (Météo-France) at Parçay-Meslay (47°26'36''N; 00°43'36''E; 13 km from the study site) in 2010 were used to illustrate meteorological conditions during the year of collections.

We collected *Culicoides* using one drop trap and one suction trap in the field and in the closed stable, and one suction trap in the largely-open-stable (Fig. 5). All traps were separated by a minimum of 30 m to minimize interference and to assess the level of endophagy of *Culicoides*. The drop trap is a host-baited trap and consisted of a rectangular cage (2.5 m wide×3 m long and 2 m high) and made of white cotton netting (<0.25 mm² mesh size). Its structure and use was described in detail by Viennet et al. [16]. The suction trap allows *Culicoides* collections without human intervention and consisted of a wooded box (48 cm wide×58 cm long and 73 cm high) equipped with a engine cooling fan at the top and an inner collection bottle rotator (model 1512, John W. Hock Company, Gainesville, FL) (Fig. 6). *Culicoides* were collected in 0.5 liter plastic collection bottles, which were filled 1/3 full with water and two drops of soap, and then were transferred in 70% ethanol.

Traps were operated during 24 consecutive hours (from 12 pm to 12 pm the next day), giving 48 collections at 30 min intervals, 6 times at each of the three 2-week periods. Each trap was baited with four south-Prealpes sheep females of 3 to 5-years old and 55 to 65 kg weight enclosed in a pen (2.5 m long×2 m wide×1.3 m high). There were no horses or cattle within 200 m from the sites during the collection sessions. The protocol was examined internally in the *Plate Forme d'Infectiologie Expérimentale* (UE1277 PFIE) by an internal group in charge of animal welfare, including veterinary surgeon and animal keepers. The protocol procedure did not cause any pain or stress, *i.e.* no injection, no biological sample, no surgery. Thus, according to the Directive 2010/63/EU on the protection of animals used for scientific purposes in Europe, it was not necessary to submit this protocol to an ethics committee and each step of the protocol was conducted with respect to the standard ethical rules: staff were qualified for animal experimentation and premises were licensed for experimentation.

In parallel, a UV-light/suction trap (manufactured by the Onderstepoort veterinary institute in South Africa) was operated during each collection session to provide an overview of *Culicoides* diversity. It was run with an 8 W UV light tube and on a 12-volt car battery, was placed at 1.5 m height from the ground on a tree and was not visible from the animal baits (Fig. 5). The insects collected with the UV-light/suction trap were stored in 70% ethanol.

References

- Combes C (2000) Selective pressure in host-parasite systems. *Journal de la Société de Biologie* 194: 19–23.
- Combes C (2010) *L'art d'être parasite: les associations du vivant*; Flammarion, editor, Paris.
- Clements AN (1999) *The biology of mosquitoes. Sensory reception and behaviour*; Oxon, editor, New York: CABI.
- Tauber CA, Tauber MJ (1981) Insect seasonal cycles: genetics and evolution. *Annual Review of Ecology and Systematics* 12: 281–308.
- Barnard BJH (1997) Some factors governing the entry of *Culicoides* spp. (Diptera: Ceratopogonidae) into stables. *Onderstepoort Journal of Veterinary Research* 64: 227–233.

Culicoides Identification

All *Culicoides* were morphologically identified under a stereomicroscope (Stemi 2000 C ZEISS) to species level based on an identification key for the Palaearctic region [36] and sorted by sex. Females were classified as nulliparous, parous [37], freshly blood-fed and gravid. When morphological identification with a stereomicroscope was not possible, individuals were dissected and identified using microscopic slide preparations (ZEISS imager A.1 fluorescence microscope).

Individuals belonging to the Obsoletus Complex (*C. obsoletus* and *C. scoticus*) were molecularly identified following the assay developed by Nolan *et al.* [38]. DNA extraction was done high-throughput using Chelex100[®] resin (200 µL/*Culicoides*) before polymerase chain reaction (PCR) [39]. Primers and PCR amplifications conditions were as described by Nolan *et al.* [38].

Statistical Analysis

Outdoor activity of *Culicoides* was compared between periods of the year to highlight influence of day length on circadian activity and was compared with climatic conditions to assess influence of meteorological parameters on host-seeking activity.

Abundance of *Culicoides* species was modeled using a Poisson mixed-effect model fitted with a method providing an adaptive Gauss-Hermite approximation to the log-likelihood. We used the session (1 to 18) as a random effect, and the location (field, closed stable and largely-open-stable), the trap (drop trap, suction trap) and the season (spring, summer and autumn) as fixed effects. We considered also interactions between location and season, as endophagic behavior may change between the different seasons [12,13]. Selection of effects and/or interactions in the abundance model was based on a likelihood ratio test, and Pearson's product-moment correlation was used as an overall test for goodness of fit.

Finally, we tested outdoor biting rates assessed by the host-baited traps with the abundance in UV-light collections using linear models.

All data analyses were performed using the R statistical package [40].

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Author Contributions

Conceived and designed the experiments: EV CG CM T. Baldet T. Balenghien. Performed the experiments: EV CG IR XA JL RV DC MR T. Balenghien. Analyzed the data: EV CG RL T. Balenghien. Contributed reagents/materials/analysis tools: LG IF RL. Wrote the paper: EV CG T. Balenghien.

- Meiswinkel R, Baylis M, Labuschagne K (2000) Stabling and the protection of horses from *Culicoides bolitinos* (Diptera: Ceratopogonidae), a recently identified vector of African horse sickness. *Bulletin of Entomological Research* 90: 509–515.
- Wilson AJ, Mellor PS (2009) Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society B-Biological Sciences* 364: 2669–2681.
- Velthuis AGJ, Saatkamp HW, Mourits MCM (2009) Financial consequences of the dutch bluetongue serotype 8 epidemics of 2006 & 2007. *Preventive Veterinary Medicine*: 11.

9. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, et al. (2012) Novel Orthobunyavirus in Cattle, Europe, 2011. *Emerging Infectious Diseases* 18: 469–472.
10. Overgaard Nielsen B, Christensen O (1975) A mass attack by biting midge *Culicoides nubeculosus* (Mg.) (Diptera, Ceratopogonidae) on grazing cattle in Denmark a new aspect of sewage discharge. *Nordisk Veterinærmedicin* 27: 365–372.
11. Meiswinkel R, Goffredo M, Dijkstra EGM, van der Ven IJK, Baldet T, et al. (2008) Endophily in *Culicoides* associated with BTV-infected cattle in the province of Limburg, South-Eastern Netherlands, 2006. *Preventive Veterinary Medicine* 87: 182–195.
12. Baldet T, Delécolle JC, Cetre-Sossah C, Mathieu B, Meiswinkel R, et al. (2008) Indoor activity of *Culicoides* associated with livestock in the bluetongue virus (BTV) affected region of Northern France during autumn 2006. *Preventive Veterinary Medicine* 87: 84–97.
13. Baylis M, Parkin H, Kreppel K, Carpenter S, Mellor PS, et al. (2010) Evaluation of housing as a means to protect cattle from *Culicoides* biting midges, the vectors of bluetongue virus. *Medical and Veterinary Entomology* 24: 38–45.
14. Carpenter S, Szmaragd C, Barber J, Labuschagne K, Gubbins S, et al. (2008) An assessment of *Culicoides* surveillance techniques in northern Europe: have we underestimated a potential bluetongue virus vector? *Journal of Applied Ecology* 45: 1237–1245.
15. Gerry AC, Monteys VSI, Vidal JOM, Francino O, Mullens BA (2009) Biting rates of *Culicoides* midges (Diptera: Ceratopogonidae) on sheep in northeastern Spain in relation to midge capture using UV light and carbon dioxide-baited traps. *Journal of Medical Entomology* 46: 615–624.
16. Viennet E, Garros C, Lancelot R, Allene X, Gardes L, et al. (2011) Assessment of vector/host contact: comparison of animal-baited traps and UV-light/suction trap for collecting *Culicoides* biting midges (Diptera: Ceratopogonidae), vectors of Orbiviruses. *Parasites & Vectors* 4: 119.
17. EFSA (2008) Bluetongue Scientific Opinion of the Panel on Animal Health and Welfare (Question No EFSA-Q-2007–201) adopted on 19 June 2008. *The EFSA Journal* 735: 1–70.
18. Barnard DR, Jones RH (1980) Diel and seasonal patterns of flight activity of Ceratopogonidae in Northeastern Colorado: *Culicoides*. *Environmental Entomology* 9: 446–451.
19. Lillie TH, Kline DL, Hall DW (1987) Diel and seasonal activity of *Culicoides* spp. (Diptera: Ceratopogonidae) Near Yankeetown, Florida, Monitored with a vehicle-mounted insect trap. *Journal of Medical Entomology* 24: 503–511.
20. van der Rijt R, van den Boom R, Jongema Y, van Oldruitenborgh-Oosterbaan MM (2008) *Culicoides* species attracted to horses with and without insect hypersensitivity. *The Veterinary Journal* 178: 91–97.
21. Calvete C, Estrada R, Miranda MA, del Rio R, Borrás D, et al. (2009) Entry of bluetongue vector *Culicoides imicola* into livestock premises in Spain. *Medical and Veterinary Entomology* 23: 202–208.
22. Anderson GS, Belton P, Belton EM (1993) A population study of *Culicoides obsoletus* Meigen (Diptera, Ceratopogonidae), and other *Culicoides* species in the Fraser Valley of British-Columbia. *Canadian Entomologist* 125: 439–447.
23. Votýpka J, Synek P, Svobodová M (2009) Endophagy of biting midges attacking cavity-nesting birds. *Medical and Veterinary Entomology* 23: 277–280.
24. Garcia-Saenz A, McCarter P, Baylis M (2011) The influence of host number on the attraction of biting midges, *Culicoides* spp., to light traps. *Medical and Veterinary Entomology* 25: 113–115.
25. Murray MD (1987) Local dispersal of the biting midge *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) in south-eastern Australia. *Australian Journal of Zoology* 35: 559–573.
26. Kettle DS, Edwards PB, Barnes A (1998) Factors affecting numbers of *Culicoides* in truck traps in coastal Queensland. *Medical and Veterinary Entomology* 12: 367–377.
27. Ninio C, Augot D, Dufour B, Depaquit J (2011) Emergence of *Culicoides obsoletus* from indoor and outdoor breeding sites. *Veterinary parasitology* 183: 125–129.
28. Zimmer J-Y, Saegerman C, Losson B, Haubruge E (2010) Breeding sites of bluetongue virus vectors, Belgium [letter]. *Emerging infectious diseases* 16: 575–576.
29. Mellor PS, Boorman J, Baylis M (2000) *Culicoides* biting midges: Their role as arbovirus vectors. *Annual Review of Entomology* 45: 307–340.
30. Kettle DS (1969) The biting habits of *Culicoides furens* (Poey) and *C. barbosai* Wirth & Blanton. I. The 24-h cycle, with a note on differences between collectors. *Bulletin of entomological research* 59: 21–31.
31. Blackwell A (1997) Diel flight periodicity of the biting midge *Culicoides impunctatus* and the effects of meteorological conditions. *Medical and Veterinary Entomology* 11: 361–367.
32. Sanders CJ, Gubbins S, Mellor PS, Barber J, Golding N, et al. (2012) Investigation of diel activity of *Culicoides* biting midges (Diptera: Ceratopogonidae) in the United Kingdom by using a vehicle-mounted trap. *Journal of Medical Entomology* 49: 757–765.
33. Kettle DS (1969) The biting habits of *Culicoides furens* (Poey) and *C. barbosai* Wirth & Blanton. II. Effects of meteorological conditions. *Bulletin of entomological research* 59: 241–258.
34. Tsutsui T, Hayama Y, Yamakawa M, Shirafuji H, Yanase T (2010) Flight behavior of adult *Culicoides oxystoma* and *Culicoides maculatus* under different temperatures in the laboratory. *Parasitology research* 108: 1575–1578.
35. Balenghien T, Cetre-Sossah C, Grillet C, Delecolle JC, Mathieu B, et al. (2008) Diurnal activity of potential bluetongue vectors in northern Europe. *Veterinary Record* 162: 323–324.
36. 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 de Doctorat d'Université mention Sciences]. Strasbourg, France: Université Louis Pasteur. 238 p.
37. Dyce AL (1969) The recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *Journal of the Australian Entomological Society* 8: 11–15.
38. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, et al. (2007) Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pubicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Veterinary Microbiology* 124: 82–94.
39. Solano P, Duvallet G, Dumas V, Cuisance D, Cuny G (1997) Microsatellite markers for genetic population studies in *Glossina palpalis* (Diptera: Glossinidae). *Acta Tropica* 65: 175–180.
40. R Development Core Team (2008) An Introduction to R: Notes on R, A Programming Environment for Data Analysis and Graphics. In: Computing RFI, editor. 3.2.1 ed. Vienna, Austria.