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1 ***Revealing potential bridge vectors for BTV and SBV: a study on Culicoides***
2 ***blood feeding preferences in natural ecosystems in Spain***

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6 ***Culicoides host-feeding preferences***

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1 **ABSTRACT**

2 Several species of *Culicoides* (Diptera: Ceratopogonidae) are vectors of pathogens,
3 such as the Bluetongue (BTV) and the Schmallenberg (SBV) viruses, that cause
4 important diseases in domestic and wild ruminants. Since wild ruminants can
5 contribute to overwintering and epizootics of both diseases, knowledge of
6 *Culicoides* host-feeding behaviour in natural ecosystems is an important parameter
7 to better understand their epidemiology. Blood engorged *Culicoides* females trapped
8 in natural areas inhabited by different wild ruminant species were genetically
9 analysed to identify host species. The origin of blood meals was identified in 114
10 females of 14 species of *Culicoides*. A total of 104 (91.1%) *Culicoides* fed on
11 mammals and 10 (8.9%) on birds. The most abundant host identified was red deer
12 (66.7%), followed by human (13%) and fallow deer (6.1%). Eleven of the 14
13 species of *Culicoides* fed exclusively on mammalian hosts. Among them, 5 are
14 mammalophilic species considered important BTV and/or SBV vectors. The results
15 of the present study confirm that *C. imicola*, *C. obsoletus*, *C. scoticus*, *C. pulicaris*
16 and *C. punctatus* fed on wild ruminants, and therefore support the hypothesis that
17 these species can act as bridge vectors by facilitating the circulation of pathogens
18 between wild and domestic ruminant communities.

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20 **KEY WORDS:** *Culicoides*, BTV, host preference, SBV, Spain, vector, wild
21 ruminants

1 INTRODUCTION

2 The genus *Culicoides* has been extensively studied due to the fact it contains species
3 that act as vectors of several diseases. There are approximately 1400 *Culicoides*
4 species in the world (Borkent 2012, 2014). The females of 96% of these species are
5 expected to be obligate blood-feeders, and in consequence, can act as vectors of
6 protozoa, filarial worms and viruses affecting birds, humans, and other mammals
7 (Mellor *et al.*, 2000). One of the most economically important pathogens transmitted
8 by *Culicoides* is Bluetongue virus (BTV), which produces an infectious non-
9 contagious disease of domestic and wild ruminants (Mellor & Wittmann, 2001).
10 *Culicoides* transmit Schmallenberg virus (SBV) to ruminants as well, which was
11 detected for the first time in Germany and the Netherlands in summer and autumn
12 2011 (De Regge *et al.*, 2012; Elbers *et al.*, 2013; Goffredo *et al.*, 2013; Larska *et al.*,
13 2013; Balenghien *et al.*, 2014). Since then, the disease has been detected in almost
14 the whole of Europe. In Spain, it has been reported to affect sheep and goats
15 (Fernández-Aguilar *et al.*, 2014). To date, six species of *Culicoides* have been
16 described as vectors for BTV and SBV in Europe: *C. obsoletus*, *C. scoticus*
17 (Carpenter *et al.*, 2006; Carpenter *et al.*, 2008a; De Liberato *et al.*, 2005; Savini *et*
18 *al.*, 2005, De Regge *et al.*, 2012; Elbers *et al.*, 2013; Goffredo *et al.*, 2013), *C.*
19 *dewulfi* (Meiswinkel *et al.*, 2007; De Regge *et al.*, 2012; Elbers *et al.*, 2013;
20 Goffredo *et al.*, 2013), *C. chiopterus* (Dijkstra *et al.*, 2008; De Regge *et al.*, 2012;
21 Elbers *et al.*, 2013), *C. pulicaris* (Caracappa *et al.*, 2003; Balenghien *et al.*, 2014),
22 and *C. imicola* (Balenghien *et al.*, 2014). *Culicoides punctatus* (Larska *et al.*, 2013)
23 and *C. nubeculosus* (Balenghien *et al.*, 2014) are considered SBV vectors, and a
24 recent study indicates that *C. newsteadi* and *C. punctatus* might act as BTV vectors
25 (Goffredo *et al.*, 2015).

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High seroprevalence for BTV and SBV antibodies in wild ruminants suggests wildlife might play an important role in the transmission of such pathogens in Europe (Falconi *et al.*, 2011; Chiari *et al.*, 2014). Moreover, it has been observed that some *Culicoides* species feeding on domestic ruminants are abundant and in close contact with wild ruminants in natural ecosystems, which suggests that these species may be acting as bridge vectors (Talavera *et al.*, 2015). To be sure of the role of these species as bridge vectors and to better understand the epidemiology of the different diseases transmitted by *Culicoides*, it is necessary to know the standing host preferences of *Culicoides* species inhabiting natural ecosystems. However, most of the studies dealing with *Culicoides* host feeding preferences in Europe have been conducted around livestock areas (e.g. Rawlings *et al.*, 2003; Bartsch *et al.*, 2009; Garros *et al.*, 2011; Viennet *et al.*, 2011; Lassen *et al.*, 2011; Ninio *et al.*, 2011; Calvo *et al.*, 2012; Pettersson *et al.*, 2013). Only recently have some studies been performed in natural ecosystems (Santiago-Alarcón *et al.*, 2013, Bobeva *et al.*, 2015), and to the best of our knowledge none of them in natural areas with an important presence of wild ruminants. While some studies have inferred *Culicoides* host preferences based on the morphology of the sensory organs present in the antennae and the palps (Blackwell 2004), direct evidence is needed to be sure of the food source of a particular species. Early studies on *Culicoides* feeding patterns were based on serological (Braverman *et al.*, 1971; Walker & Davie, 1971; Nevill & Anderson, 1972; Boakye *et al.*, 1999) and immunological assays (Blackwell *et al.*, 1994; Blackwell *et al.*, 1995). However, these methods allowed the identification of a limited number of potential vertebrate host species. Recently, molecular techniques have been developed, which have increased the accuracy of host

1 identification at species level (Votýpka *et al.*, 2009; Bobeva *et al.*, 2015; Martínez
2 de la Puente *et al.*, 2015; Slama *et al.*, 2015).

3 Given the sparse knowledge about *Culicoides* hosts in natural ecosystems, the main
4 goal of the present study is to identify by means of molecular techniques the
5 vertebrate species on which *Culicoides* feed in these ecosystems. Knowing which
6 vertebrate species the different species of *Culicoides* found in natural ecosystems
7 feed on, will shed light on which species of *Culicoides* can act as bridge and
8 enzootic vectors for *Culicoides* borne pathogens, and therefore on the mechanisms
9 behind the spillover of BT and SB from domestic to wild ruminants (and vice-
10 versa). In addition, this information could indirectly indicate which reservoirs are
11 significant in associated vector-borne diseases.

13 **MATERIALS AND METHODS**

15 **Sampling**

16 *Culicoides* were trapped between years 2009-2010 in seven private Spanish areas of
17 land characterized by their distinctive bioclimatic features and wild ruminant
18 communities (S1 Table). Bioclimatic variables and altitude were obtained from a
19 climatic atlas of the Iberian Peninsula (Ninyerola *et al.*, 2005). Landscape variables
20 were obtained from the Global Environment Monitoring database (Global Land
21 Cover 2000). The distribution of ruminant species in Spain was obtained from the
22 atlas of land mammals in Spain (Palomo *et al.*, 2007). Three CDC black light traps
23 (John W. Hock Company, Gainesville, FL, USA) were placed close to water points
24 used as troughs for wild ruminants. The traps were located at a distance of more than 1
25 km from domestic ruminants and the distance between traps was about 15 m. Traps

1 were switched on at dusk and off at dawn the following day for three consecutive
2 nights once a month during summer and autumn for two consecutive years. In
3 Spain, seven species of wild ruminants are present, red deer (*Cervus elaphus*) being
4 the most abundant, fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*),
5 mouflon (*Ovisaries musimon*), Spanish ibex (*Capra pyrenaica hispanica*), Pyrenean
6 chamois (*Rupicapra pyrenaica*) and aoudad (*Ammotragus lervia*).

8 **Morphological and molecular identification**

9 Trapped insects were killed by drowning in the capture containers with soapy water,
10 and then stored in 70% ethanol. *Culicoides* midges were morphologically identified
11 under a stereomicroscope (Nikon SMZ) to species-group level according to wing
12 pattern. An accurate morphological identification was performed of at least one
13 female of each species. Specimens were dissected using sterilized ultrafine
14 tweezers, mounted on slides with Canada Balsam solution, and identified to species
15 level according to their morphology (Campbell & Pelham-Clinton, 1960; Kremer
16 1965; Kremer *et al.*, 1973; Delécolle 1985; Glukhova 1989) using a Nikon Eclipse
17 E200 light microscope. Once females of the *Obsoletus* complex had been grouped
18 according to wing pattern, species identification was performed genetically. Briefly,
19 DNA was isolated from wild caught individuals and used as a template in a PCR
20 reaction targeting the gene marker cytochrome oxidase I (COI), as described in
21 Pagès & Sarto i Monteys, 2005 and Nolan *et al.*, 2007.

25 **Blood identification**

1 Blood engorged females were used for DNA extraction. The abdomen of each blood
2 engorged female was dissected with sterilized ultrafine tweezers. The abdomen
3 served as a template for DNA extraction using DNeasy Blood and Tissue kit
4 (Qiagen), in accordance with the manufacturer's recommendations. DNA was used
5 for amplification of a fragment of COI gene using a nested PCR with degenerated
6 primers, as described in Alcaide *et al.* 2009. DNA purified PCR products were
7 sequenced on both strands using Big Dye Terminator version 3.1 cycle sequencing
8 kit (Applied Biosystems) and analysed on an ABI PRISM 3730 Automated
9 sequencer (Applied Biosystems).

10 DNA sequences were edited using Bioedit sequence alignment editor software
11 (version 5.0.9. for Windows; Hall, 1999). Fasta files were used for nucleotide
12 BLAST (Basic Local Alignment Search Tool) for host species identification.

14 **RESULTS**

16 **Collection of biting midges**

17 A total of 102,693 *Culicoides* specimens were collected during the study period.

18 Less than 1% of collected *Culicoides* were blood engorged females (n=264).

19 Engorged females were collected at all sampling sites except in Boumort where
20 collections were scarce (0.1% total captures; S1 Table). Among the 264 blood
21 engorged females, genetic characterization of blood-meal origin was successful in
22 114 (44%) of them, probably due to an enzymatic degradation of the blood within
23 the digestive tract of the midge. Identification of hosts selected by *Culicoides* to
24 feed on their blood was achieved for 14 *Culicoides* species. Among them, 47 were

1 obtained from *C. obsoletus*, 13 from *C. scoticus*, 10 from *C. punctatus* and 9 from
2 *C. imicola* (Table 1).

3 4 **Blood host identification**

5 *Culicoides* fed on 12 different host species, 6 mammals and 6 birds. Eleven of the
6 14 *Culicoides* species exclusively had bloodmeals of mammalian origin, two had
7 blood only from avian hosts, and one species was found to be fed both on mammals
8 and birds (Table 1).

9 At the individual level, among the 114 females in which blood origin was
10 successfully identified, 95 (83.5%) fed on species of wild vertebrates, 15 (13%) on
11 humans and 4 (3.5%) on cattle. All *Culicoides* with cattle blood were collected in La
12 Almoraima, the closest sampling site to livestock facilities (one kilometre; S1
13 Table). At a taxonomic level, 104 (91%) of the 114 bloodmeals identified were of
14 mammals and 10 (9%) of birds (Table 1). In bloodmeals, the most frequently
15 identified host was red deer (*Cervus elaphus*, 66.7%), human (*Homo sapiens*, 13%)
16 and fallow deer (*Dama dama*, 6.1%). The most frequent avian hosts were blackbird
17 (*Turdus merula*, 3.5%) and hoopoe (*Upupa epops*, 1.8%) (Table 1).

18 In all areas the most abundant ruminant host was red deer (*Cervus elaphus*) except
19 for El Juanar where it was Spanish ibex (*Capra pyrenaica hispanica*) (S1 Table).

20 The most abundant wild ruminant of each zone according to local censuses was
21 detected as the preferred host for *Culicoides*, except in La Morera where only
22 human and hoopoe blood was detected (Table 1).

23 24 **DISCUSSION**

1 Recent studies highlight that most *Culicoides* species are able to feed on several
2 vertebrate species, but usually present preferences for mammals or for birds
3 (Martínez de la Punte *et al.*, 2015), demonstrating that many species of *Culicoides*
4 are not generalist (Santiago-Alarcon *et al.*, 2012). The results obtained here support
5 those from the aforementioned previous studies, since *Culicoides* specimens
6 collected at natural ecosystems were found to feed mainly on one or other of the two
7 groups, mammals or birds. Nevertheless, mammalophilic and ornithophilic
8 *Culicoides* species might feed, respectively, on different mammal or bird species
9 opportunistically. Host selection generally seems to reflect host availability
10 (Pettersson *et al.*, 2013).

11 *Culicoides* species previously described as strict mammal bloodfeeders based on
12 morphological traits (Talavera *et al.*, 2015) contained exclusively blood of
13 mammalian origin. Similarly, species previously described as ornithophilic feeders
14 (Talavera *et al.*, 2015) had avian blood except *C. univitattus*, which was found to
15 have only fed on humans. However, all these specimens were trapped in the same
16 locality and during the same period. *Culicoides festivipennis* mainly fed on birds;
17 however one of the seven bloodmeals identified in this species was of human origin.
18 Thus, although according to our data *C. festivipennis* seems to prefer birds, they also
19 partially support studies describing it as a generalist feeder (Santiago-Alarcon *et al.*,
20 2012). *Culicoides* species with generalist preferences are of special interest because
21 they could promote the emergence of new diseases (Santiago-Alarcon *et al.*, 2012).
22 The genetic identification of bloodmeals allowed us to report four new *Culicoides*
23 hosts for the first time in Europe: two wild ruminants, fallow deer (*Dama dama*) and
24 Spanish ibex (*Capra pyrenaica*), and two birds, *Sylvia cantillans* and *Upupa epops*
25 (for purposes of comparison see Martínez de la Puente *et al.*, 2015).

1 Knowledge about vector host preferences is critical to better understand the
2 epidemiology of vector borne diseases (Mukabana *et al.*, 2002; Lyimo & Ferguson,
3 2009). Thus, the opportunistic or selective host tendencies of a vector may affect the
4 spread of a disease (Burkot 1988). The *Culicoides* host preferences are dependent on
5 the intrinsic features of each *Culicoides* species, as well as on environmental factors
6 such as host availability (Lyimo & Ferguson, 2009; Burkot 1988). However, most
7 studies of *Culicoides* host preferences have usually been performed in farms or rural
8 areas with livestock because of the economic importance of *Culicoides* transmitted
9 pathogens for livestock production (Rawlings *et al.*, 2003; Bartsch *et al.*, 2009;
10 Garros *et al.*, 2011; Viennet *et al.*, 2011; Lassen *et al.*, 2011; Ninio *et al.*, 2011;
11 Pettersson *et al.*, 2013). The main BTV and SBV vectors (*C. imicola*, *C. obsoletus*,
12 *C. scoticus*, *C. pulicaris* and *C. punctatus*) collected in this study were found to feed
13 exclusively on mammals, mainly wild ruminants (70/82; Table 1). The present study
14 reports for the first time the origin of *C. imicola* bloodmeals molecularly identified
15 in Europe. In Africa *C. imicola* was described feeding mainly on horses, cattle and
16 sheep (Logan *et al.*, 2010), and also on humans, birds and dogs (Slama *et al.*, 2015).
17 The results of the present study supports the hypothesis that *C. imicola*, *C.*
18 *obsoletus*, *C. scoticus*, *C. pulicaris* and *C. punctatus* have the potential to act as
19 bridge vectors and circulate pathogens at the interface between wild and domestic
20 ruminant communities. Interestingly, two thirds of the bloodmeals identified in the
21 study were of red deer, whose population density is similar to that of domestic
22 livestock in some areas of Europe. Red deer is susceptible to BTV and might carry
23 the virus asymptotically for long periods, thus acting as a potential reservoir
24 candidate (Rodríguez-Sánchez *et al.*, 2010). In fact, previous studies found that

1 there were more BT cases in domestic ungulates in areas where red deer was present
2 (Falconi *et al.*, 2011).

3 There are some species described as ornithophilic or with unclear feeding habits as
4 *C. parroti*, *C. saevus* and *C. yemenensis* (Talavera *et al.*, 2015), with low catches,
5 but feeding exclusively on wild ruminants in natural ecosystems. These species have
6 been poorly studied to date because of sparse collection in domestic ruminant farms.
7 Different authors have pointed out that UV light-traps do not accurately reflect
8 proportions of *Culicoides* biting in the field (Logan *et al.*, 2010; Venter *et al.*, 2012),
9 suggesting that these traps could underestimate the assessment of BTV risk
10 (Carpenter *et al.*, 2008b; Gerry *et al.*, 2009). *C. parroti* belongs to the subgenus
11 *Monoculicoides*, which includes the main BTV vectors in the US (*C. sonorensis*, *C.*
12 *variipennis* and *C. occidentalis*), and has a wide distribution through Europe to
13 Russia (Augot *et al.*, 2013; Grogan & Lysyk, 2015). Thus, its potential role in the
14 epidemiology of BTV and SBV should be further investigated.

15 A total of 13% of engorged *Culicoides*, belonging to seven different species, were
16 found to bite on humans, which concurs with previous studies which have reported
17 that common mammalophilic *Culicoides* species in Europe, including arbovirus
18 vectors, occasionally feed on humans (Slama *et al.*, 2015; Garros *et al.*, 2011;
19 Santiago-Alarcon *et al.*, 2012; Carpenter *et al.*, 2013). Attacks on humans mainly
20 occur when preferred hosts such as livestock or wildlife are either scarce or absent.

21 Worldwide, the role of *Culicoides* in the transmission of zoonotic arboviruses is
22 currently unclear, but thought to be limited (Carpenter *et al.*, 2013).

23 Field seroprevalence and experimental infections suggest that wild ruminants are
24 susceptible to BTV and SBV infection (Falconi *et al.*, 2011; Chiari *et al.*, 2014;
25 Ruiz-Fons *et al.*, 2008; Ruiz-Fons *et al.*, 2014; García *et al.*, 2009; García *et al.*,

1 2011; Rodríguez-Sánchez *et al.*, 2010; Linden *et al.*, 2012; Laloy *et al.*, 2014;
2 Fernández Aguilar *et al.*, 2014). Molecular analyses of bloodmeals of *Culicoides*
3 species collected in natural ecosystems confirm that the major putative BTV and
4 SBV vectors in Europe (*C. imicola*, *C. obsoletus*, *C. scoticus*, *C. pulicaris* and *C.*
5 *punctatus*) are mostly mammalophilic and bite more frequently on wild ruminants.
6 Therefore, these species might act as bridge vectors leading the spillover of both
7 diseases between domestic and wild ruminants in epizootic areas.

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Table 1. Blood meal sources (vertebrates hosts) of engorged *Culicoides*. **Pro.**, Proaza; **P.P.**, Puig la Penya; **Q.M.**, Quintos de Mora; **L.M.**, La Morera; **E.J.**, El Juanar; **L.A.**, La Almoraima

Species	Mammals						Birds						% Blood engorged
	<i>Capra pyrenaica</i>	<i>Cervus elaphus</i>	<i>Dama dama</i>	<i>Homo sapiens</i>	<i>Sus scrofa</i>	<i>Bos taurus</i>	<i>Cyanistes caeruleus</i>	<i>Delichon urbica</i>	<i>Erithacus rubecula</i>	<i>Sylvia cantillans</i>	<i>Turdus merula</i>	<i>Upupa epops</i>	
<i>C. cataneii</i>							L.A. 1					L.M.1	1,8
<i>C. circumscriptus</i>												L.A.1	1,8
<i>C. festivipennis</i>				L.M.1				Pro.1	Q.M.1	Q.M.1	Q.M.2	L.A. 1	6,1
<i>C. imicola</i>		L.A. 6					L.A. 3						7,9
<i>C. newsteadi</i>		L.A. 1	L.A. 2	L.A. 1			L.A. 1						4,4
<i>C. obsoletus</i>	E.J. 1	Pro. 26 P.P.10 Q.M. 2 L.A. 4		Pro. 1 Q.M. 2	Pro. 1								41,1
<i>C. parroti</i>		Q.M. 3 L.A. 1	L.A.1										4,4
<i>C. pulicaris</i>		P.P. 2	P.P. 1	Pro.1									3,5
<i>C. punctatus</i>		P.P. 2 Q.M. 6	P.P. 2										8,8
<i>C. saevus</i>		Q.M. 2											1,8
<i>C. scoticus</i>		Pro. 4 P.P.1 L.A.4	P.P.1	L.A. 3									11,3
<i>C. subfagineus</i>		L.A. 1											0,9
<i>C. univitatus</i>				L.A. 5									4,4
<i>C. yemenensis</i>		L.A. 1		L.A. 1									1,8
% Blood engorged	0,9	66,7	6,1	13	0,9	3,5	0,9	0,9	0,9	0,9	3,5	1,8	

S1 Table. Data summary of ecological variables and characterization of the sampling sites (Palomo *et al.*, 2007; Global Land Cover 2000). **A**, altitude; **AP**, annual precipitation; **LT**, mean low temperature of the coldest month; **HT**, mean high temperature of warmest month (Ninyerola *et al.*, 2005)

Sampling site	Geographical variables Coordinates	Bioclimatic Classification	A(m)	Bioclimatic variables			Environment near the sampling site		Ruminants in sampling place (more abundant - less abundant)	Blood engorged <i>Culicoides</i> % total
				HT(°C)	LT(°C)	API(mm)	Domestic ruminants (distance in km)			
Proaza	43°12' N 06°03' W	Temperate oceanic submediterranean	349	25	0	1000	Farm (4,5)	red deer, roe deer	29,8	
R.N.C.Boumort	42°12' N 01°06' E	Temperate hyperoceanic	1276	25	-7,5	900	Free domestic livestock (1)	red deer, fallow deer, roe deer, chamois	0	
Puig la Penya	42°31' N 02°32' E	Mediterranean pluvisesional oceanic	228	27,5	2,5	1000	Farm (2)	red deer, fallow deer, mouflon	16,7	
Quintos de Mora	39°23' N 04°06' W	Mediterranean pluvisesional oceanic	718	35	0	482	Farm (10)	red deer, fallow deer, roe deer	17,6	
La Morera	38°54' N 04°16' W	Mediterranean pluvisesional oceanic	707	35	2,5	500	Farm (4)	red deer, mouflon, aoudad	1,8	
El Juanar	36°34' N 04°53' W	Mediterranean pluvisesional oceanic	870	27,5	2,5-5	850	Farm (5)	spanish ibex	0,88	
La Almoraima	36°27' N 05°43' W	Mediterranean pluvisesional oceanic	45	30,5	7,5	955	Farm (4)	red deer, fallow deer, roe deer, mouflon	33,3	