New species of the genus Culicoides *(Diptera Ceratopogonidae) for Tunisia, with detection of Bluetongue viruses in vectors*

Soufien Sghaier[1*], Salah Hammami[2], Maria Goffredo[3], Mounira Hammami[1], Ottavio Portanti[3], Alessio Lorusso[3], Giovanni Savini[3] and Jean Claude Delécolle[4]

[1] *Université Tunis El Manar, Institut de la Recherche Vétérinaire de Tunisie, 20 Rue Djebel Lakdhar, 1006 La Rabta, Tunis, Tunisia.*
[2] *Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, 2020 Sidi Thabet, Tunisia.*
[3] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, Teramo, Italy.*
[4] *Université de Strasbourg, Faculté de Médecine, Institut de Parasitologie et Pathologie Tropicale, 3 rue Koeberlé, 67000 Strasbourg, France.*

[*] *Corresponding author at: Institut de la Recherche Vétérinaire de Tunisie, 20 Rue Djebel Lakdhar, 1006 La Rabta, Tunis, Tunisia. Tel.: +216 55335503, e-mail: sghaiersoufien@yahoo.fr.*

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Keywords
Bluetongue virus, *Culicoides*, Entomology, Epizootic haemorrhagic disease virus, Surveillance, Tunisia, Vectors.

Summary
Bluetongue virus (BTV) and Epizootic haemorrhagic disease virus (EHDV) are double-stranded RNA orbiviruses of the *Reoviridae* family. Bluetongue virus and EHDV infect domestic and wild ruminants and they are transmitted by biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae). Since 1999, BTV outbreaks have occurred in Tunisia and 4 serotypes, BTV2, BTV1, BTV4 and BTV3, were involved in 2000, 2006, 2009, and 2016, respectively. Epizootic haemorrhagic disease was detected for the first time in Tunisia and in other Northern African countries in 2006. These incursions have caused considerable economic losses. Our study had the goal to describe diversity, distribution, and seasonal dynamics of *Culicoides*. Fourteen sampling sites were chosen throughout the country and 2-night trapping of midges was performed monthly from June 2006 to July 2008. A total of 11,582 *Culicoides* specimens were collected from 336 light traps, comprising 25 species, of which 7 were identified for the first time in Tunisia, increasing to 35 the total number of *Culicoides* species now reported in this country. Twenty-three pools of parous females belonging to the *Culicoides imicola* and *Culicoides kingi* were tested for detection of BTV and EHDV by molecular assays. Both BTV1 and BTV4 were detected in *C. imicola*.

Specie del genere Culicoides *(Diptera Ceratopogonidae) e presenza del virus della Bluetonge in Tunisia*

Parole chiave
Bluetongue virus, *Culicoides*, Entomologia, Virus della malattia emorragica epizootica, Sorveglianza, Tunisia, Vettori.

Riassunto
I virus della Bluetongue (BTV) e della malattia emorragica epizootica (EHDV) sono orbivirus a doppio filamento di RNA della famiglia delle *Reoviridae*. Questi virus infettano ruminanti domestici e selvatici e sono trasmessi da insetti morsicatori del genere dei *Culicoides* (Diptera: Ceratopogonidae). In Tunisia si rilevano focolai di BTV dal 1999; nel 2000, nel 2006, nel 2009 e nel 2016 sono stati coinvolti rispettivamente i sierotipi BTV2, BTV1, BTV4 e BTV3. Il virus della malattia emorragica epizootica è stato invece individuato per la prima volta in Tunisia e nei Paesi del Nord Africa nel 2006. Queste incursioni hanno causato notevoli danni economici. Il nostro studio ha avuto l'obiettivo di descrivere la diversità, la distribuzione e le dinamiche stagionali dei *Culicoides*. Sono stati scelti quattordici siti di campionamento in tutto il paese e da giugno 2006 a luglio 2008 sono state eseguite mensilmente campagne di cattura di due notti. Con 336 trappole di luce sono stati catturati complessivamente 11.582 esemplari di *Culicoides*, appartenenti a 25 specie diverse; 7 di esse venivano rilevate in Tunisia per la prima volta e hanno portato a 35 il numero totale di specie qui accertate. Per verificare la presenza di BTV e EHDV, sono stati analizzati 23 gruppi di femmine gravide di *Culicoides imicola* e *Culicoides kingi* con indagini molecolari; nei *Culicoides imicola* sono stati rilevati i ceppi di BTV1 and BTV4.

Introduction

Bluetongue (BT) and Epizootic Haemorrhagic Disease (EHD) are infectious arthropod-borne viral diseases that affect both domestic and wild ruminant species (MacLachlan 1994, Campbell and St George 1986). Bluetongue virus (BTV) and EHD virus (EHDV) are double-stranded RNA viruses of the genus *Orbivirus*, *Reoviridae* family. They are mainly transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae) (Mellor *et al.* 2000). Thus, the distribution of the BTV and EHD Reoviridae and the intensity of infection depend on the repartition and abundance of *Culicoides*.

Between 1999 and 2002, BTV serotype 2 epizootics occurred in Tunisia (Ben Fredj *et al.* 2003, Hammami 2004) and in 2006 and 2009, Tunisian authority notified the incursion of 2 other serotypes: BTV1 and BTV4, respectively. More recently, other BT outbreaks – due to serotype 1 in 2011 and serotype 4 in 2013 – have been reported (Lorusso *et al.* 2013, Sghaier *et al.* 2014). In November 2016, serotype 3 made an unexpected incursion (Sghaier *et al.* 2017) and has spread mainly to the East coast of the country.

Like BTV, also the distribution of EHDV depends on the distribution of competent *Culicoides* vectors. According to the reported cases, EHDV lies approximately between latitudes 35°S and 49°N (Savini *et al.* 2011). Recently, clinical forms in cattle have been reported in countries surrounding the Mediterranean Basin – including Morocco, Algeria, and Tunisia – where the emergence of EHDV serotype 6 has been reported (Ben Dhaou *et al.* 2016).

Several entomological studies on biting midges have been conducted to establish an inventory of the species circulating in Tunisia (Chaker 1981, Chaker *et al.* 2005, Hammami *et al.* 2008 a, b, Slama *et al.* 2014, Slama *et al.* 2016). However, these studies are limited in time and in space.

In the present study, we describe the findings of an entomological surveillance in 14 sites in non-desert areas. Different pools of parous females belonging to the *Culicoides imicola* and *Culicoides kingi* species were tested for the presence of EHDV and BTV genomes. *Culicoides kingi* was selected based on the findings of a previous study in Sudan, where this species has been found to be infected by EHDV (Mellor *et al.* 1984, Sellers 1984).

Materials and methods

Study area

Fourteen representative sites were selected. They were situated within a framework of squares of 45 × 45 km in non-humid and non-desert area of Tunisia (Table I and Figure 1). The choice of the site depended on 2 criteria: the presence of identifiable housed cattle, belonging to the Tunisian office of public lands (OTD) and the availability of an alternating 220V current power supply. The 14 sites were included in 3 bioclimatic regions: 3 in the sub-humid region, 5 in the semi-arid region, and 6 in the arid zone.

Traps and insect collections

Two-night catches per site were performed monthly

Table I. *Location and data about the 14 sites of collected biting midges (2006-2008) in Tunisia.*

Trap code	Farm locality	Governorate	Nb and race of animals	Positioning of Trap / Animals	Latitude; Longitude	Altitude
MA	Mateur	Bizerte	40 cattle	Inside / 2m	37°3'27.24"N; 9°36'45.08"E	17 m
BA	Borj Amri	Mannouba	30 cattle	Outside / 2m	36°41'36.25"N; 9°52'36.99"E	61 m
TA	Takelsa	Nabeul	50 cattle	Outside / 2m	36°51'56.22"N; 10°44'23.49"E	320 m
TH	Thibar	Béja	50 cattle	Outside / 5m	36°31'48.58"N; 9°5'58.10"E	44 m
EN	Enfidha	Sousse	30 cattle	Outside / 2m	36°7'28.52"N; 10°23'4.75"E	17 m
SR	Sers	Le Kef	60 cattle	Outside / 15m	36°6'52.39"N; 8°55'37.92"E	52 m
RM	Rmila/Siliana Sud	Siliana	50 cattle	Outside / 15m	36°1'26.41"N; 9°24'11.18"E	5 m
AL	Alem/Sebikha	Kairouan	100 cattle	Outside / 2m	35°53'44.36"N; 10°3'5.60"E	472 m
MO	Monastir	Monastir	15 cattle 20 sheep	Outside / 2m	35°44'11.57"N; 10°48'53.36"E	32 m
OD	Oued darb/ Kasserine Sud	Kasserine	50 cattel	Inside / 15m	35°11'2.76"N; 8°50'33.45"E	543 m
TO	Touila/Sidi bouzid ouest	Sidi Bouzid	40 cattle	Inside / 10m	35°1'32.90"N; 9°27'6.76"E	341 m
CH	Chaal/Thina	Sfax	30 cattle	Outside / 20m	34°40'59.67"N; 10°38'17.97"E	647 m
SN	Sened	Gafsa	12 cattle 30 sheep	Outside / 15m	34°33'14.24"N; 9°11'45.95"E	476 m
KE	Kettana/Mareth	Gabes	60 cattle	Outside / 10m	33°43'56.21"N; 10°14'33.04"E	36 m

using black UV-light traps manufactured by the Onderstepoort Veterinary Institute (OVI, South Africa). Samples were taken from July 2006 to June 2008. Traps were set 1 hour before sunset and were positioned outdoors as near as possible to cattle and suspended at a height of 1.5-2 m above the ground. Traps were removed at about 8 am the next day. Insects were collected in a beaker containing 200-250 mL of water and 3-4 drops of detergent, as a wetting agent. Each catch was transported to the laboratory, then covered and preserved in 70% ethanol for further study. *Culicoides* were sorted according to wing patterns, using stereoscopic microscope and taxonomic keys, and subsequently confirmed by mounting specimens on microscope slides (Wirth and Marston 1968, Delécolle 1985). For virus detection, adult parous females of *C. imicola* and *C. kingi* were selected in 2011 according to the abdomen pigmentation (Dyce 1969), and divided in pools of maximum 15 individuals. A total of 20 pools of *C. imicola* (n = 233) and 3 of *C. kingi* (n = 24) were sorted from 16 collections, conducted in 6 localities in 2006 and 2007 (Table II). The Minimum Infection Rate (MIR) was calculated by dividing the number of positive pools by the number of midges tested.

Figure 1. *Map showing the location of 14 sampling sites for* Culicoides *in Tunisia during entomological surveillance between July 2006 and June 2008.*

RNA extraction for BTV and EHDV detection

The insect pools were homogenized with the pellet pestle motor (Kontes, Vineland, New Jersey, USA) in a 2 mL tubes filled with 300 µL of PBS. The RNA was extracted from supernatant using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer´s instructions.

Real-Time qRT-PCR for BTV detection and serotyping

The detection of BTV (Hofmann *et al.* 2008) was carried out using a real-time reverse transcriptase polymerase chain reaction (qRT-PCR) kit (*i.e.* RT-PCR Kit SuperScript III Platinum® One-Step Quantitative RT-PCR System; Invitrogen, Carlsbad, California, USA). Used primers and TaqMan probe target the NS3 region of BTV and the NS5-2 region of the West Nile virus (WNV) (Table III). The WNV was used as the internal positive control of the reaction. Interpretation of results was based on the cycle threshold (Ct) of FAM and VIC fluorescence: Ct (FAM) < 50 Positive, Ct (FAM) = 50; Ct (VIC) < 37 Negative, Ct (FAM) = 50; Ct (VIC) ≥ 37 Inhibition.

Type specific real-time qRT-PCR assays were carried out targeting the segment 2 of European and Mediterranean BTV types, namely BTV-1, 2, 4, 6, 8, 9, 11, and 16. These reactions were assessed using kits supplied by LSI (TermoFisher scientific, Waltham, Massachusetts, United States), according to the manufacturer's instructions.

qRT-PCR for EHDV detection

The EHDV detection was performed using LSI VetMax Epizootic Haemorrhagic Disease Virus kit (Laboratoire Service International, Lissieu, France) according to the manufacturer's instructions.

Results

A total of 11,582 *Culicoides* specimens were collected from 336 light traps, comprising 25 species of which 7 have been identified for the first time in Tunisia: *Culicoides obsoletus, Culicoides submaritimus, Culicoides univitatus, Culicoides subfasciipennis, Culicoides indistinctus, Culicoides santonicus,* and *Culicoides fasciipennis* (Table IV).

Culicoides imicola was the most prevalent *Culicoides*

Table II. *Identification of* Culicoides *pools caught in Tunisia, site and date of sampling, number of* Culicoides *per pool and result of Bluetongue virus (BTV) and Epizootic haemorrhagic disease virus (EHDV) genome detection.*

Site	Date of sampling	Nb of *Culicoides* per pool	qRt-PCR Bluetongue	Bluetongue serotype	CT	qRt-PCR EHDV
Monastir	12/11/2006	4 *C. imicola*	Neg	-	-	Neg
Takelsa	6/12/2006	9 *C. imicola*	Pos	BTV4	28	Neg
Alem	9/11/2006	17 *C. imicola*	Neg	-	-	Neg
Thibar	29/9/2006	18 *C. imicola*	Neg	-	-	Neg
Alem-1	5/10/2006	15 *C. imicola*	Neg	-	-	Neg
Alem-2	5/10/2006	15 *C. imicola*	Neg	-	-	Neg
Alem-3	5/10/2006	15 *C. imicola*	Neg	-	-	Neg
Alem-4	5/10/2006	4 *C. imicola*	Neg	-	-	Neg
Alem-5	5/10/2006	2 *C. kingi*	Neg	-	-	Neg
Takelsa	2/10/2006	2 *C. kingi*	Neg	-	-	Neg
Enfidha	10/9/2007	5 *C. imicola*	Pos	BTV1	21	Neg
Takelsa	9/11/2006	10 *C. imicola*	Inhib	-	-	Neg
Thibar	13/9/2006	19 *C. imicola*	Pos	BTV1	19	Neg
Enfidha	22/10/2006	20 *C. kingi*	Neg	-	-	Neg
Monastir	13/8/2007	4 *C. imicola*	Neg	-	-	Neg
Takelsa	2/10/2006	12 *C. imicola*	Neg	-	-	Neg
Monastir	15/9/2007	8 *C. imicola*	Neg	-	-	Neg
Enfidha-1	9/10/2006	15 *C. imicola*	Neg	-	-	Neg
Enfidha-2	9/10/2006	15 *C. imicola*	Neg	-	-	Neg
Enfidha-3	9/10/2006	15 *C. imicola*	Neg	-	-	Neg
Enfidha-4	9/10/2006	15 *C. imicola*	Neg	-	-	Neg
Kasserine	7/9/2007	3 *C. imicola*	Inhib	-	-	Neg
Kasserine	22/12/2006	1 *C. imicola*	Inhib	-	-	Neg

Table III. *Primers and probe for the region NS3 of Bluetongue virus (BTV) and for the NS5-2 region of West Nile virus (WNV).*

Primers and probe TaqMan for the NS1 region of BTV	
BTNS3 probe TaqMan	5'-FAM- ARG CTG CAT TCG CAT CGT ACG C –BHQ1-3'
Primer BTNS3-F	5'- TGG AYA AAG CRA TGT CAA A -3'
Primer BTNS3-R	5'- ACR TCA TCA CGA AAC GCT TC -3'
Primers and probe TaqMan for the NS5-2 region of WNV	
NS5-2 probe TaqMan	5'-VIC-CCA ACG CCA TTT GCT CCG CTG – TAMRA-3'
Primer NS5-2-F	5'-GAA GAG ACC TGC GGC TCA TG -3'
Primer NS5-2-R	5'-CGG TAG GGA CCC AAT TCA CA -3'

species in Tunisia with 25% of the total captured specimens, followed by *Culicoides cirumscriptus*, *Culicoides paolae*, and *Culicoides newsteadi* with 23%, 15%, and 13%, respectively (Figure 2).

Seven species of *Culicoides* were detected in all sites: *C. imicola*, *C. circumscriptus, Culicoides newsteadi*, *Culicoides paolae, Culicoides sahariensis, Culicoides cataneii*, and *Culicoides jumineri* (Table V).

The distribution rate of different *Culicoides* species varies from one site to another. Indeed, some minority species present in one site may be a majority in another one. This was observed for *C. jumineri* species, which rose from 1% to 54% in Takelsa and in Kettana, respectively. Midges of the Obsoletus complex were rare with the exception of the Thibar site, where they reached 10% of the total captured specimens (Figure 3).

Three sites have a high diversity of *Culicoides* species: Thibar, Mateur, and Takelsa with respectively 22, 21, and 18 identified species (Table V).

Our results demonstrated that along the 2 years of study, the population dynamics of *Culicoides* showed a peak in the month of September. However, the period, when biting midges are more active, is from June to October (Figure 4).

The flight dynamics of male midges show a major peak of activity in April 2006 for *C. paolae, C. cirumscriptus*, and *C. imicola*. However, in 2007, the male flight activity was spread over time with series of small peaks for *C. cicumscriptus, C. sahariensis, C. paolae*, and *C. imicola*. Peaks were also registered in July, August, September, and October (Figure 5).

The percentages of males caught range from 42.7% for *C. paolae* to by 24%, 23%, and 21% for *C. circumscriptus, C. univitatus*, and *C. sahariensis*, respectively (Figure 6).

Table IV. *Updated checklist of* Culicoides *species recorded in Tunisian fauna.*

Reported species found	Newly recorded species (This study)
1 - *Culicoides (Avaritia) imicola* Kieffer, 1913 (B)	19 - *Culicoides (Avaritia) obsoletus* Meigen, 1818
2 - *Culicoides (Beltranmyia) circumscriptus* Kieffer, 1918 (A)	20 - *Culicoides (Oecacta) santonicus* Callot, Kremer, Rault & Bach, 1966
3 - *Culicoides (Culicoides) newsteadi* Austen, 1921 (B)	21 - *Culicoides (Sensiculicoides) indistinctus* Khalaf, 1961
4 - *Culicoides (Culicoides) punctatus* Meigen, 1804 (C)	22 - *Culicoides (Sensiculicoides) submaritimus* Dzhafarov, 1962
5 - *Culicoides (Monoculicoides) puncticollis* Becker, 1903 (A)	23 - *Culicoides (Sensiculicoides) univittatus* Vimmer, 1932
8 - *Culicoides (Oecacta) longipennis* Khalaf, 1957 (A)	24 - *Culicoides (Silvaticulicoides) fascipennis* Staeger, 1839
7 - *Culicoides (Oecacta) marcleti* Callot, Kremer & Basset, 1968 (A)	25 - *Culicoides (Silvaticulicoides) subfasciipennis* Kieffer, 1919
9 - *Culicoides (Oecacta) sahariensis* Kieffer, 1923 (A)	**Reported species not found**
6 - *Culicoides (Pontoculicoides) saevus* Kieffer, 1922 (A)	26 - *Culicoides (Monoculicoides) parroti* Kieffer, 1922 (A)
10 - *Culicoides (Remmia) kingi* Austen, 1912 (A)	27 - *Culicoides (Monoculicoides) riethi* Kieffer, 1914 (A)
11 - *Culicoides (Sensiculicoides) cataneii* Clastrier, 1957 (A)	28 - *Culicoides (Oecacta) corsicus* Kremer, Leberre & Beaucournu-Saguez, 1971 (A)
12 - *Culicoides (Sensiculicoides) heteroclitus* Kremer & Callot, 1965 (A)	29 - *Culicoides (Oecacta) semimaculatus* Clastrier, 1958 (D)
13 - *Culicoides (Sensiculicoides) jumineri* Callot & Kremer, 1969 (A)	30 - *Culicoides (Oecacta) sergenti* Kieffer, 1921 (D)
15 - *Culicoides (Sensiculicoides) kurensis* Dzhafarov, 1960 (C)	31 - *Culicoides (Sensiculicoides) gejgelensis* Dzhafarov, 1964 (A)
16 - *Culicoides (Sensiculicoides) maritimus* Kieffer, 1924 (A)	32 - *Culicoides (Sensiculicoides) griseidorsum* Kieffer, 1918 (A)
17 - *Culicoides (Sensiculicoides) odiatus* Austen, 1921 (A)	33 - *Culicoides (Sensiculicoides) lailae* Khalaf, 1961 (A)
14 - *Culicoides (Sensiculicoides) pseudopallidus* Khalaf, 1961 (A)	34 - *Culicoides (Sensiculicoides) langeroni* Kieffer, 1921 (A)
18 - *Culicoides paolae* Boorman, Mellor & Scaramozzino, 1996 (B)	35 - *Culicoides pseudolangeroni* Kremer, Chaker & Delécolle, 1981 (A)

Reference of record in Tunisia: (A) Chaker & Kremer 1982; (B) Chaker *et al.* 2005; (C) Hammami *et al.* 2008; (D) Slama *et al.* 2016.

Among the Obsoletus complex, all 39 males captured were *C. obsoletus s.s.*, whereas no specimens belonging to the species *C. scoticus* were identified.

Out of the 23 pools of *Culicoides* analysed, 3 pools of *C. imicola*, collected from 3 sites, were positive for BTV by qRT-PCR. Serotyping revealed the presence of serotype 1 in September 2006 in the region of Thibar and serotype 4 in December of the same year in the site of Takelsa. One pool of *C. imicola* captured in September 2007 from the site of Enfidha was also positive for BTV1.

The Ct values were 19, 28, and 21 for Thibar, Takelsa, and Enfidha, respectively.

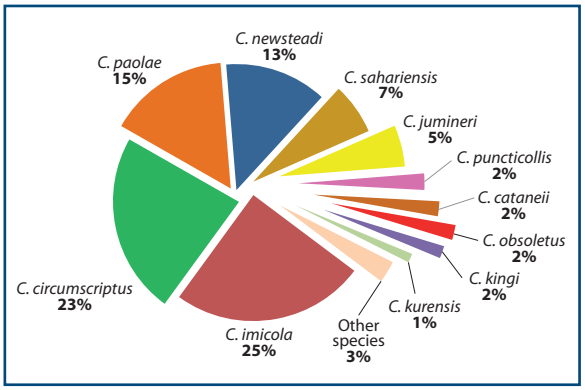

Figure 2. *Global distribution of different species of* Culicoides *captured in Tunisia during entomological surveillance between July 2006 and June 2008.*

The Minimum Infection Rate of *C. imicola* in Thibar was 2.7% (1/37), in Takelsa 3.4% (1/29) in 2006, and in Enfidha 20% (1/5) in 2007.

However, the 3 pools of *C. kingi* were negative for BTV. All *Culicoides* pools were negative for EHDV (Table II).

Discussion

Our results demonstrate a large variability of the distribution of different species of *Culicoides* depending on the geographical location. The most widespread species in cattle farms were *C. imicola*, *C. circumscriptus, C. paolae*, and *C. newsteadi*.

The difference in the diversity of species caught by site is not related to the importance of the catch but to the presence of wetlands and abundant vegetation nearby farms. Indeed, the 3 sites that had the best *Culicoides* diversity belong to sub-humid bioclimatic region.

The Obsoletus complex is among the most abundant biting midges in Europe (Baldet *et al.* 2006, Mehlhorn *et al.* 2009), where it is a potential vector of BT (Savini *et al.* 2005, De Liberato *et al.* 2005). In particular, the Obsoletus complex has recently been found positive in the field for BTV1 and BTV4 in Italy (Goffredo *et al.* 2015). This is in discordance with our findings in the present study.

In Tunisia, the distribution of this species is limited to some sites with relatively low abundance.

Table V. *Occurrence of the* Culicoides *spp. in the different sites of trapping, different grey shades indicate the number of* Culicoides.

	MA	BA	TA	TH	EN	SR	RM	AL	MO	OD	TO	CH	SN	KE	Σ
C. imicola	+++	+++	++++	+++	+++	+	+	++++	+++	+++	+++	+	+	+	14
C. circumscriptus	+++	+++	++++	+++	++++	+++	+++	++++	+++	++++	++	++	+	+	14
C. newsteadi	+++	+++	+++	+++	+++	++	++	+++	+++	++	++	+	+	+	14
C. paolae	++	++	+++	++++	+++	++	++	+++	++++	++	+++	+	++	+	14
C. sahariensis	++	++	+++	+++	++	++	++	++	+	+	+	+	+	++	14
C. cataneii	+	+	++	++	+	+	+	+	+	+	+	+	+	+	14
C. puncticollis	+	+	++	+	+	++	++	++	+	+	+		+		12
C. kingi	+	++	+	+	+++	+	+	+	+	+	++		+		12
C. seavus	+			+	+	+	++	+		+					7
C. jumineri	++	++	++	++	+	++	+	++	+++	+	++	+	+	+++	14
C. kurensis	++	+	+	+	+	+		+						+	8
C. pseudopallidus	+	+	+	+		+	+		+	+	+			+	10
C. marcleti				+									+		2
C. heteroclitus	+		+	+		+									4
C. longipennis			+	+											2
C. odiatus	+		+	+	+	+									5
C. punctatus	+			+					+					+	4
C. maritimus			+						+					+	3
C. submaritimus	+	+	+						+			+		++	6
C. obsoletus	++	+	+	+++			+		+					+	7
C. indistinctus	+			+											2
C. univitatus	++			+											2
C. fasciipennis				+											1
C. subfasciipennis	+		+	+											3
C. santonicus	+														1
Σ	21	*13*	18	22	12	14	12	11	14	11	10	8	10	13	

++++ = > 300; +++ = > 100; ++ = > 20; + = ≤ 20.

The Thibar site, where the Obsoletus complex constituted 10% of caught specimens, is characterised by an ecosystem similar to that found in the Southern part of Europe.

The relative abundance of males in collections may indicate nearby breeding sites.

Among *Culicoides* species collected in this study, 3 have not been reported in both Algeria and Morocco: *C. paolae, C. fascipennis,* and *C. submaritimus. Culicoides paolae* is the most abundant and widespread *Culicoides* species on the Maltese islands (Goffredo *et al.* 2004).

We confirmed the presence of BTV4 by viral RNA detection in a pool of *C. imicola* 3 years before the official notification about the circulation of this serotype in Tunisia (in 2009)[1]. This result is in accordance with the identification of BTV4 in Tunisian sheep in 2007 (Lorusso *et al.* 2013). We, also, detected BTV1 genome in a *C. imicola* pool from September 2006 in the region of Thibar, just before the appearance of the first outbreak due to this serotype in November of the same year. This result shows the importance of the establishment of an entomological surveillance coupled with detection of BTV during the peak of activity of *C. imicola*.

The number of positive specimens by pool of parous females of *C. imicola* ranges from 5 to 19 with relatively low Ct. The low Ct values, which indicate high amounts of viral RNA, suggest that the tested insects may harbour viable virus (Veronesi *et al.* 2013). The detection of BTV genome in *Culicoides* after 4 years of conservation in ethanol 70% encourages us to perform other molecular investigation on the other species in our collection.

This study allowed for the identification of 7 new *Culicoides* species for the Tunisian Fauna and 5 *Culicoides* species confirmed or suspected to be vectors of BTV and/or EHDV.

Besides *C. imicola*, at least other 4 species reported in this study have been associated to BTV or other arboviruses elsewhere, namely *C. obsoletus, C. newsteadi, C. punctatus,* and *C. kingi*. Therefore, regular entomological surveys are important to detect the various BTV vectors and to understand

[1] http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapEventSummary&reportid=8773.

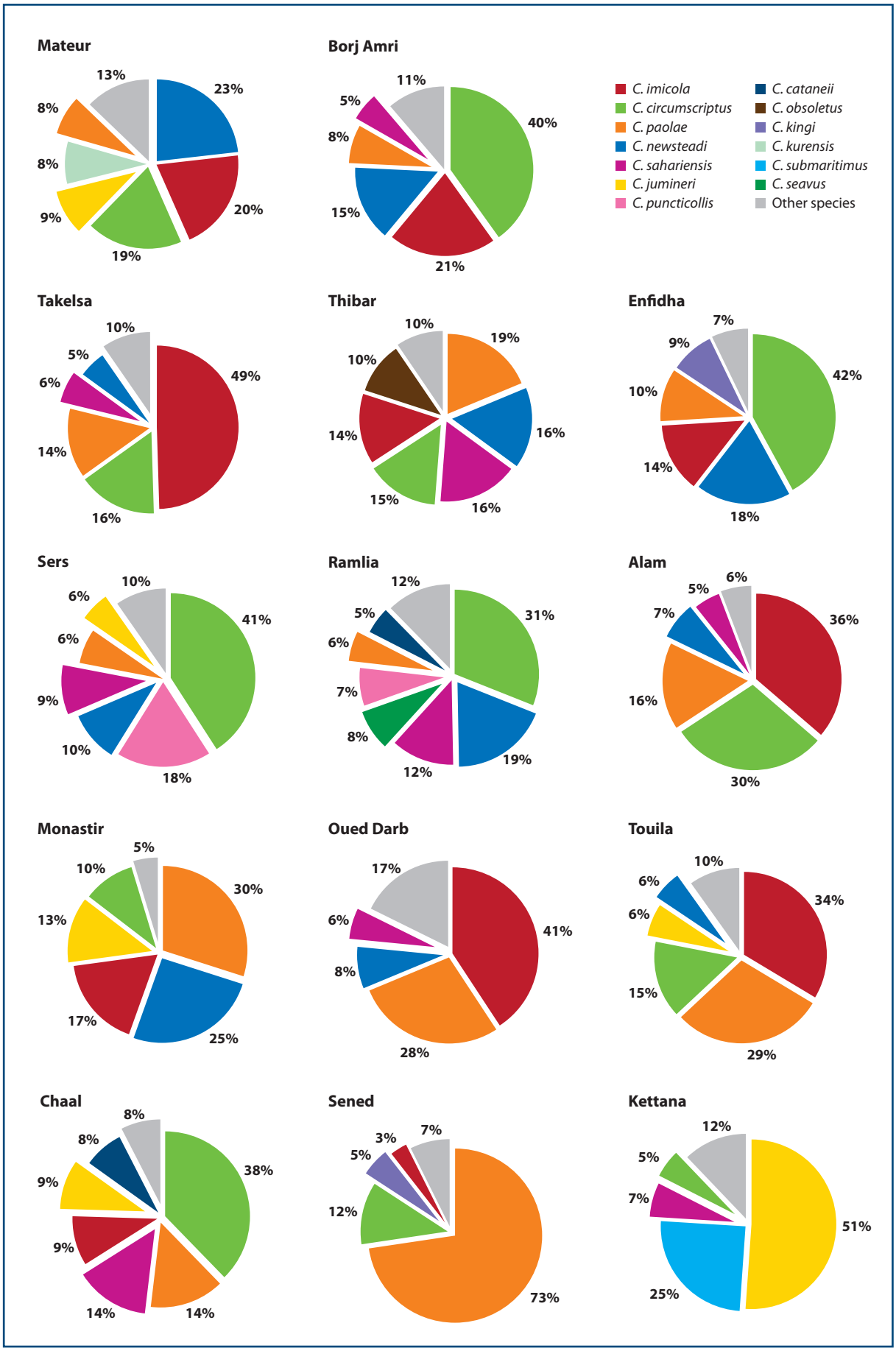

Figure 3. *Repartition of different species of* Culicoides *in function of sites between July 2006 and June 2008 in Tunisia.*

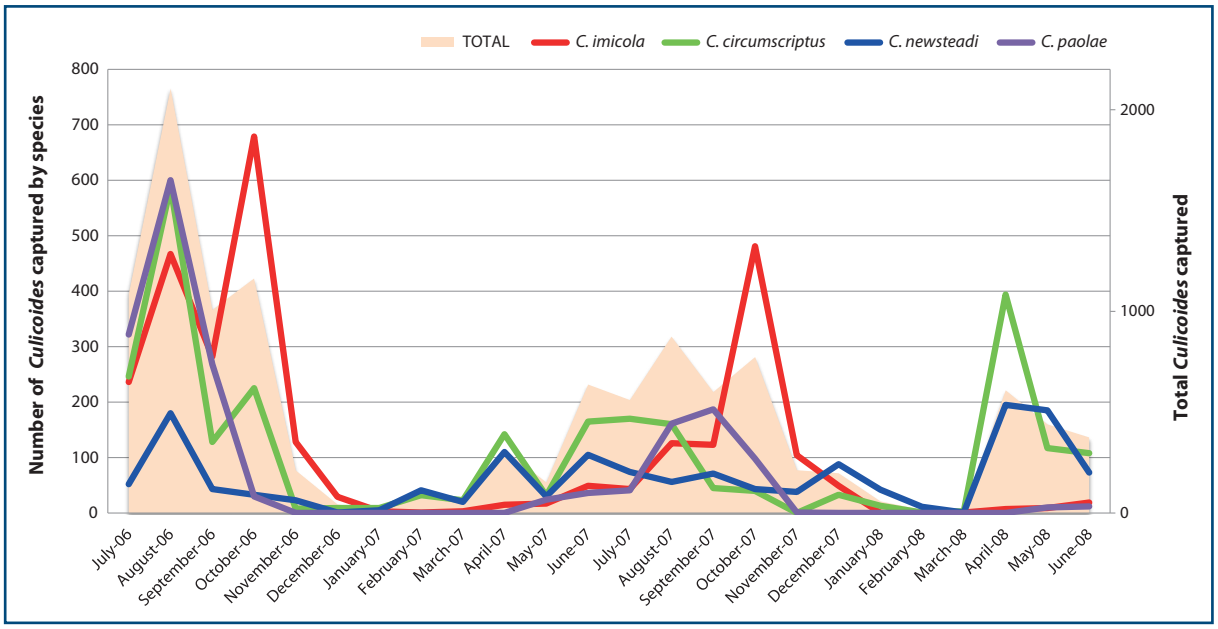

Figure 4. *Seasonality of* C. imicola, C. cirumscriptus, C. paolae *and* C. newsteadi *between July 2006 and June 2008 in Tunisia.*

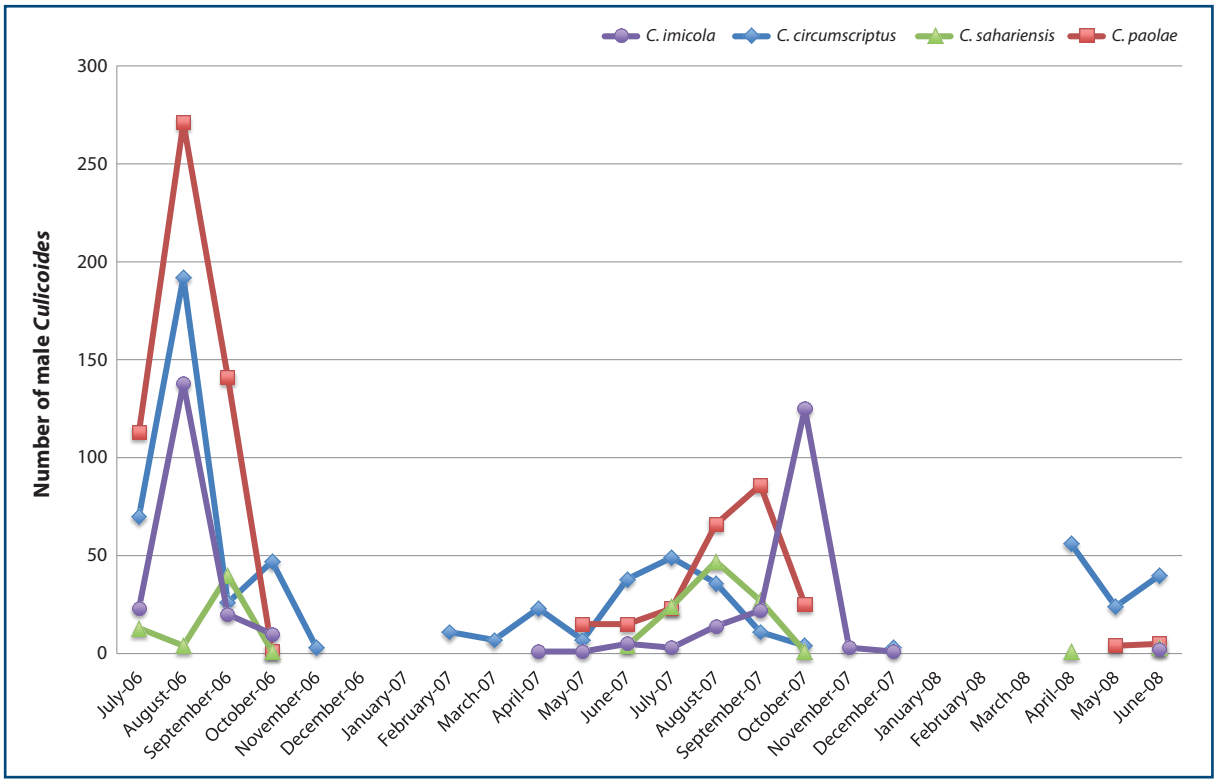

Figure 5. *Seasonality of males* C. imicola, C. cirumscriptus, C. paolae *and* C. newsteadi *between July 2006 and June 2008 in Tunisia.*

the mechanisms of orbiviruses perpetuation in endemic areas.

This study showed not only the importance of coupling active monitoring of BTV and entomological survey of its vectors nationally, but also the relevance of timely sharing information with other Mediterranean countries. The implementation of international projects involving these countries would improve our knowledge on the origins, spread, and pathways of movement for individual virus lineages and even for individual genome segments within the virus population. Exploring genetic relationships, gene flow, and microbiome composition within vector populations around

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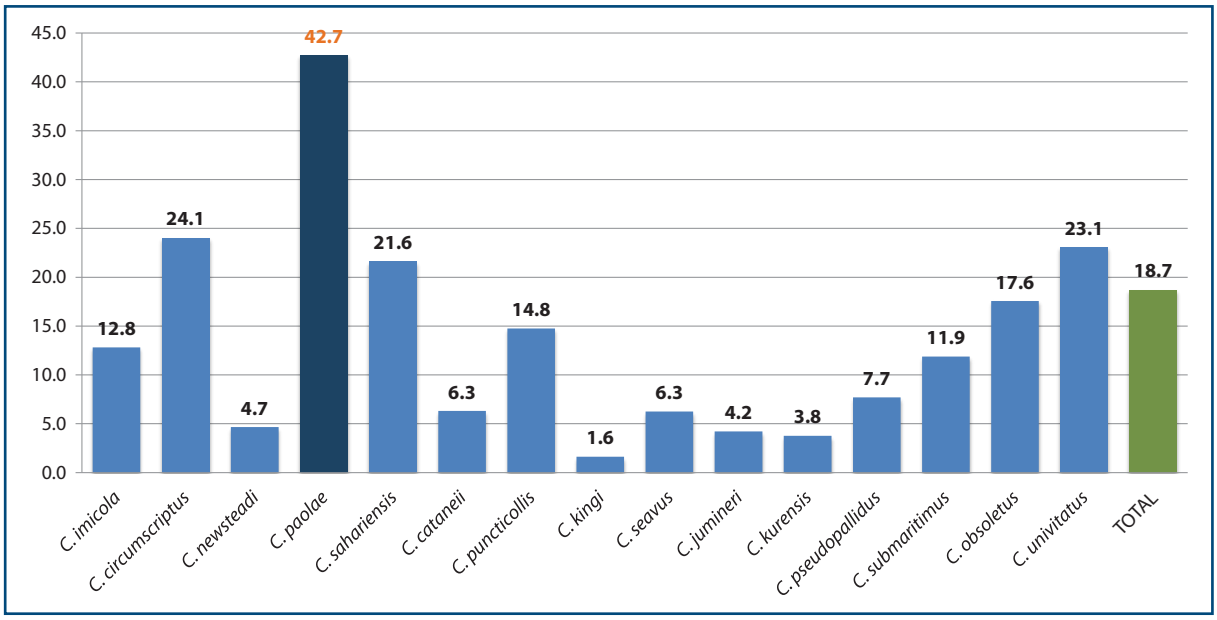

Figure 5. *Percentage of males caught per species during entomological surveillance between July 2006 and June 2008 in Tunisia.*

the Mediterranean Basin and the comparison of the findings with data obtained for the incidence and movement of BTV strains can generate further information about the regional spread and relatedness of *Culicoides* populations in different regions. Finally, the implementation and sharing of a common strategy for monitoring the disease would facilitate epidemiological analyses.

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