UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

PHENOTYPIC AND GENETIC CHARACTERIZATION OF *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) IN PORTUGAL AND COMPARISON OF THE EFFECT OF PYRETHROID INSECTICIDES IN THEIR CONTROL

DAVID WILSON RUSSO RAMILO

Orientadores: Doutora Isabel Maria Soares Pereira da Fonseca de Sampaio

Doutor Javier Lucientes Curdi

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Sanidade Animal

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2016

To my immortal grandparents, Helena Ramilo and Joaquim Ramilo,

To my beloved parents, Lúcia Russo and Luis Ramilo,

To my adorable sister, Raquel Reis,

To my godchildren, Sara Rodrigues, Tiago Reis and Sofia Reis,

To my closest family,

To my soulmate, Joana Simão,

To my most dear friends,

To Alpha and Omega (as source of knowledge…)

Acknowledgements

I would like to thank my supervisor, Professor Isabel Pereira da Fonseca, who tirelessly helped me at any time, giving the necessary advice and wisdom for all matters concerning both doctoral work and life. It was for me a pleasure to develop the present work under your supervision, together with all the friendship and trust shown, hoping that our collaboration can give more results in the near future. Thank you also for listening me when the world seems to vanish beneath my feet.

My special thanks to my co-supervisor, Professor Javier Lucientes Curdi, who kindly shared his experience and knowledge during the development of this doctoral work, and also for his quick help when time was running out.

Thanks to the coordinator of the National Entomologic Surveillance Program (NESP) for *Culicoides*, Professor Fernando Boinas, and to Direção-Geral de Alimentação e Veterinária (DGAV) for both 2010 and 2011 financial support and the opportunity to study *Culicoides* biting midges during and after the abovementioned program. Thanks to all the people who together collaborated with the NESP since 2005, in both laboratorial and data analysis.

I would also like to thank Professor Graça Alexandra-Pires and Dr. Telmo Nunes (FCUL) for their friendship, patience, availability, advice and help on scanning electron microscopy images for Chapters 2 and 5, hoping that our collaboration still be productive in the near future.

Thanks to: Doctor Elisabete Silva for her PCR support (in Chapter 2), sharing of knowledge and friendship, M.Sc. Telmo Nunes (FMV) for his availability in statistical evaluation (in Chapters 3 and 4); to Professor Manuela Oliveira for the opportunity to show part of my work to her students, for precious help during critical nerves and friendship; finally, to Professor Berta São Braz for her total trust in my person and friendship. It is and it will always be a pleasure to work with you all.

Thanks to Professor Jean-Claude Delécolle and Ph.D. Bruno Mathieu from University of Strasbourg and Ph.D. Claire Garros and her team from CIRAD, Montpellier, who received me with open arms and were extremely kind and available for me during three weeks. Thank you for sharing your facilities and laboratory material to perform my work. Thanks to Ph.D. Roger Venail from EID Méditerranée, Montpellier, France, who kindly provided *Culicoides* biting midges exposed to insecticides for the work performed in Chapter 5. Thank you all for your eternal friendship and sharing of knowledge.

Also from Montpellier, I would like to thank Doctor Christine Le Roux and Doctor Alain for their hospitability, friendship and trust during my French journey. You were very important during those days and my memory will always be with you.

My thanks go also for: Dr. Lidia Gomes, who readily showed her help and knowledge in all matters concerning laboratorial work, and also for her friendship; M.Sc. Marcos Santos, for his all-the-time availability, precious drawings and photo editing, PCR help and advice and for precious and rare friendship both as roommate and laboratory colleague; M.Sc. Ana Valente for image scaling, precious opinions and special friendship; M.Sc. Solange Pacheco, M.Sc. Rita Ribeiro and M.Sc. Sara Madeira for their availability and help during these four years of intense work from both sides, as well as for their special friendship; M.Sc. Cátia Marques for her unconditional help, mutual work, eternal friendship and huge laughs/deep thoughts during our car travels – your existence in my life is essential.

I cannot forget all the precious help given from my colleagues and students during these four years concerning insect mounting and preparation of laboratorial solutions – my deep and sincere thanks to all of you.

Thanks to all the people, who, together, constitute the Faculty of Veterinary Medicine, for their friendship, smiles, trust, knowledge, help, inspiration and source of strength in all the moments, for they are my second family. Thanks to CIISA and Faculty of Veterinary Medicine for all the financial support during this doctoral work.

A special thanks to a big friend of mine, Dr. Ana Lúcia Ferreira, who picked up all the little pieces that were sparse in my soul and bring it all together for a better comprehension of the world and of myself. Thank you for saving my life once.

Thanks to my soulmate, Joana Simão, for her precious help in this work, patience, comprehension, love and trust.

Finally, thanks to my family for all the trust and support during my entire life and also as a source of comfort and love when things go wrong.

Financial Support

This study was supported by:

National Entomologic Surveillance Program of *Culicoides* biting midges, vectors of Bluetongue virus, in national territory, funded by Direção Geral de Veterinária/Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa.

Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa (FMV-ULisboa), through the Projects UID/CVT/00276/2013 and Pest-OE/AGR/UI0276/2014, both funded by FCT.

FCT – Fundação para a Ciência e Tecnologia (FCT), through the fellowship SFRH/BD/77268/2011.

VectorNet: A European network for sharing data on the geographic distribution of arthropod vectors, transmitting human and animal disease agents, through Project OC/EFSA/AHAW/2013/02.

This work was partially funded by the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Ministère en charge de l'Agriculture (France) and by the convention Cherchuer d'Avenir 2011 from Languedoc-Roussillon region (France).

Phenotypic and genetic characterization of *Culicoides* (DIPTERA: CERATOPOGONIDAE) in Portugal and comparison of the effect of pyrethroid insecticides in their control

Abstract

Culicoides genus is of major importance in animal and human health since hematophagous females are vectors of several pathogens, like viruses (Bluetongue, African Horse Sickness and Schmallenberg) and filarial nematodes, among others. As a consequence, female biting midges are responsible for huge economical losses worldwide. A deeper knowledge of *Culicoides* fauna present in each country and their ecological preferences is required, so different control strategies can be applied efficiently.

The present work was based on *Culicoides* species captures during the National Entomologic Surveillance Program for Bluetongue disease in mainland Portugal and Azores and Madeira archipelagos (2005-2013), using miniature CDC light traps, and in 2015, with OVI traps, in the framework of VectorNet European network.

Biting midges were evaluated phenotypically and genetically, showing the variation of *Culicoides* species in Portuguese territory. Twenty-two *Culicoides* species were mentioned for the first time in Portugal, including *C. dewulfi* and *C. montanus* in mainland Portugal and species of Obsoletus group in the islands of Azores archipelago where they were never reported, as well as a description of *Culicoides paradoxalis*, a new species for science. Moreover, a detailed study focused the morphology, genetics and ecology of Obsoletus group, showing that the distribution of those species was unequal in mainland Portugal. Plus, a redefinition of the 3rd palpus segment length/width ratio and spermathecae size intervals, aiming Obsoletus group species identification, together with the reference of anatomical aberrations in these species, was performed, allowing the reduction of errors during midges studies. An identification key for all known Portuguese midges was also created.

In this thesis, elaboration of risk assessment maps based on the association of some abiotic variables with the occurrence of *C. imicola*, *C. pulicaris*, *C. punctatus*, *C. newsteadi* and species from Obsoletus group in mainland Portugal were also performed.

Finally, evaluation of *C. imicola* morphological modifications in sensorial organs localized in the 3rd palpus segment, used for host detection, was performed after an assay with pyrethroid insecticides (permethrin and deltamethrin) at different concentrations, showing complete destruction of sensorial organs with probable feeding implications.

The results presented in this scientific work contributed to a better knowledge of *Culicoides* genus in Portugal, being some of them of worldwide relevance.

Keywords: *Culicoides*, phenotypic, genetic, pyrethroid, Portugal.

Caracterização fenotípica e genética de *Culicoides* (DIPTERA: CERATOPOGONIDAE) em Portugal e comparação do efeito de inseticidas piretróides no seu controlo

Resumo

Os insetos do género *Culicoides* (Diptera: Ceratopogonidae) possuem uma grande importância em Saúde Animal e Humana, uma vez que as fêmeas hematófagas são vetores de vários agentes patogénicos, como vírus (da Língua Azul, da Peste Equina Africana, da Doença Hemorrágica Epizoótica e de Schmallenberg), filarídeos, entre outros. Consequentemente, as fêmeas do género *Culicoides* são responsáveis por elevadas perdas económicas a nível mundial. Um conhecimento profundo da fauna de *Culicoides* presente em cada país é necessário, assim como as suas preferências ecológicas, de modo a que possam ser aplicadas de modo mais eficaz diferentes estratégias de controlo.

Uma das doenças que nos últimos anos afetou gravemente os ovinos em Portugal foi a Doença da Língua Azul. O primeiro foco desta doença em Portugal foi reportado em julho de 1956, afetando os animais da região sul do território abaixo do rio Tejo, excluindo a região algarvia, tendo sido causado pelo serótipo 10 (BTV-10). O país foi considerado livre da doença em 1960 depois da aplicação de uma vacina em ovinos. Nesse período de 4 anos, a doença matou mais de 179 mil ovinos.

A Peste Equina Africana foi detetada em Portugal em Agosto de 1989 em Castro Marim, tendo-se alastrado pelo Alentejo, ao longo da zona fronteiriça. As medidas de controlo incluiram o estudo entomológico de insetos do género *Culicoides*, vacinação massiva dos cavalos, burros e mulas, aspersão de inseticidas nas cavalariças e estábulos e divulgação de informação aos proprietários dos animais sobre prevenção. Até ao final de 1989 a doença vitimou 202 cavalos na zona do rio Guadiana, tendo sido Portugal considerado livre da doença em 1992.

Após um silêncio epizoótico de 44 anos, a doença da Língua Azul voltou a surgir em território nacional, nas regiões centro-oeste e sul, em novembro de 2004, sendo desta vez causada pelo serótipo BTV-4. EM 2005, foi criado o Programa Entomológico de Vigilância Nacional para a doença da Língua Azul em Portugal Continental e nos arquipélagos dos Açores e da Madeira (DGV/FMV, 2005-2013) com o objetivo de conhecer a distribuição de *Culicoides* em território português, reportando às autoridades competentes a presença de espécies com capacidade vetorial, por forma a que se pudesse atuar em tempo real e prevenir a dispersão da Língua Azul em caso de um surto da mesma.

Em setembro de 2007, o serótipo BTV-1 foi detetado num concelho do Alentejo perto da fronteira com Espanha. Entre setembro e dezembro de 2007, o serótipo 1 (BTV-1) foi assinalado nas regiões do centro e sul de Portugal continental, tendo-se expandido para o

norte do território desde 2008 e mostrando uma diferente distribuição quando comparada com a dos serótipos BTV-4 e BTV-10.

As campanhas de vacinação contra os serótipos 4 (2005-2008) e 1 (2007-2010) do vírus da doença da Língua Azul, em ovinos e bovinos, contribuíram para o controlo da emergência e dispersão da doença. Em 2013, o serótipo BTV-4 reemergiu no nosso país, tendo sido adotadas medidas de controlo da doença, que passaram pela vacinação obrigatória dos ovinos e facultativa dos bovinos na região do Algarve contra o referido serótipo. A vacinação contra o mesmo serótipo foi permitida na região do Alentejo como medida profilática.

Finalmente, em setembro de 2015, e após um silêncio epizoótico de 3 anos, o serótipo 1 foi novamente detetado na região do Alentejo, nos concelhos de Serpa, Moura e Barrancos. Presentemente, o serótipo 1 encontra-se presente em todo o território português enquanto que o BTV-4 encontra-se em circulação apenas na região do Algarve.

Durante o Programa Entomológico de Vigilância Nacional para a doença da Língua Azul em Portugal Continental e nos arquipélagos dos Açores e da Madeira (2005-2013), com recurso a armadilhas do tipo CDC, e em 2015 com armadilhas OVI, no âmbito do grupo de trabalho europeu VectorNet, foram realizadas capturas de insetos do género *Culicoides* que permitiram a realização do presente trabalho.

Os insetos capturados foram analisados fenotípica e geneticamente, mostrando a variação das espécies de *Culicoides* em território português. *C. imicola* foi a espécie mais capturada, representando 70,92% do total estimado de insetos capturados. Seguidamente, surgiu *C. achrayi* (10,34%), *C. punctatus* (9,34%), espécies do grupo Obsoletus (4,93%) e *C. newsteadi* (1,93%) como as espécies mais capturadas. *C. pulicaris*, espécie vetor do vírus da Língua Azul, perfez 0,08% do total estimado de insetos capturados (N=4.384.502).

A distribuição espacial das espécies de *Culicoides* em Portugal Continental apresentou padrões diferentes. Assim, enquanto que algumas espécies deste género estavam dispersas por todo o território (*e.g.*, *C. achrayi*, *C. punctatus*), outras localizaram-se preferencialmente nas regiões norte e centro (*e.g.*, *C. deltus*, *C. heliophilus*), diversas nas regiões centro e sul (*e.g.*, *C. nubeculosus*, *C. sahariensis*) e algumas foram capturadas consistentemente em regiões específicas (*e.g.*, *C. impunctatus* e *C. lupicaris*).

Vinte e duas espécies de *Culicoides* foram mencionadas pela primeira vez em Portugal: *C. alazanicus*, *C. deltus*, *C. dewulfi*, *C. heliophilus*, *C. jumineri* near *C. bahrainensis*, *C. jurensis*, *C. kingi*, *C. lupicaris*, *C. malevillei*, *C. montanus*, *C. paolae*, *C. picturatus*, *C. remmi*, *C. riebi*, *C. santonicus*, *C. semimaculatus*, *C. simulator* e *C. subfagineus* em Portugal continental, *C. obsoletus*, *C. scoticus* nas ilhas do arquipélago dos Açores onde ainda não tinham sido referidas e *C. circumscriptus* e *C. newsteadi* em todas as ilhas do mesmo arquipélago, com exceção das Flores e do Corvo. Para além destas, foi realizada a descrição de uma nova espécie para a ciência, *Culicoides paradoxalis*.

Outro objetivo deste trabalho focou a morfologia, a genética e a ecologia do grupo Obsoletus, mostrando que a distribuição destas espécies é diferente em Portugal continental. Entre as quatro espécies presentes, *C. dewulfi* foi apenas identificada em três explorações no Norte do território português. As restantes três espécies do grupo Obsoletus encontravam-se presentes em todo o país, sendo *C. obsoletus* a espécie mais prevalente no Norte, Centro Sul e Sul de Portugal continental, enquanto que *C. scoticus* foi a espécie mais prevalente na região Centro Norte. Esta espécie foi menos comum que *C. obsoletus* e *C. montanus* nas regiões Centro Sul e Sul. Estas três espécies estão bem adaptadas ao território de Portugal Continental, com a exceção da região Centro Sul.

A correta identificação de espécies do complexo Obsoletus nem sempre é possível devido às suas elevadas semelhanças morfológicas. Nesse sentido, foi realizado um estudo com base em várias estruturas anatómicas destes insetos, que levaram à redefinição dos intervalos relativos ao rácio comprimento/largura do 3.º segmento do palpo e ao comprimento das espermatecas. Para além disso, a menção de várias aberrações anatómicas nestas espécies (fossetas sensoriais aberrantes, fossetas sensoriais duplas, segmentos do palpo fundidos, segmentos do palpo com alterações morfológicas, número irregular de espermatecas, espécimens com genitália masculina e simultaneamente com espermatecas, comprimento do 3.º artículo do palpo desigual dentro do mesmo exemplar, flagelómeros antenares fundidos e aberrantes) permitirá uma redução nos erros associados ao estudo destes insetos.

Uma chave de identificação para todas as espécies de *Culicoides* presentes em Portugal foi também elaborada durante este estudo que incidiu sobre cerca de 93.100 exemplares deste género.

Nesta tese, a produção de mapas de análise de risco baseados na associação de algumas variáveis abióticas climáticas (temperatura média no período mais seco e no período mais húmido) e edáficas (territórios artificializados, áreas agrícolas e agroflorestais, florestas e meios naturais e seminaturais, zonas húmidas e corpos de água) com a ocorrência de *C. imicola, C. pulicaris, C. newsteadi, C. punctatus* e espécies do grupo Obsoletus em Portugal Continental foi também efetuada. A partir destes mapas observou-se que a probabilidade de capturar *C. imicola* é maior nas regiões a sul do rio Tejo e na Beira Baixa, enquanto que *C. pulicaris* ocorre com maior frequência nas regiões do norte de Portugal continental, na linha costeira e em zonas com elevada altitude. A probabilidade de ocorrência de *C. newsteadi* é semelhante à de *C. imicola*, embora aquela espécie possa ser capturada em regiões ligeiramente mais a norte que esta última. *C. punctatus* pode ocorrer com uma probabilidade maior que 50% em qualquer parte de Portugal Continental e em quase todas as estações do ano. As espécies do grupo Obsoletus surgem nas regiões do norte de Portugal continental, estando estas espécies praticamente ausentes na região Centro Sul durante o outono e inverno. A distribuição do grupo Obsoletus é aproximadamente a oposta de *C. imicola*.

Finalmente, a avaliação de modificações morfológicas dos órgãos sensoriais localizados no 3.º segmento do palpo de *C. imicola*, usados na deteção do hospedeiro, foi realizada através de microscopia eletrónica de varrimento após um ensaio com inseticidas piretróides (permetrina e deltametrina) em diferentes concentrações. Este estudo revelou a completa destruição dos órgãos sensoriais, com uma provável influência na alimentação destes insetos.

Tendo em conta o impacto económico associado aos agentes patogénicos que transmitem, aliado à descoberta recente de novos serótipos do vírus da Língua Azul na Europa (BTV-25 e BTV-27), os resultados apresentados neste trabalho científico evidenciam e sustentam a importância do estudo entomológico dos insetos do género *Culicoides*.

Palavras-chave: *Culicoides*, fenótipo, genética, piretróides, Portugal.

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Book Chapters

Alexandre-Pires, G., Ramilo, D., Diaz, S., Meireles, J., Boinas, F. & Pereira da Fonseca, I. (2010). Investigating morphological structures of *Culicoides* from Obsoletus complex by using Scanning Electron Microscopy and Composed Optical Microscopy. In A. Méndez-Vilas & J.D. Álvarez, *Microscopy: Science, Technology, Applications and Education*. (pp. 792-802). Badajoz, Spain: Formatex Research Center.

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List of Abbreviations

1-Octen-3-Ol	Octenol
16S rRNA	16 Svedberg Ribosomal Ribonucleic Acid, a component of the 30 Svedberg small prokaryotic ribosomal subunit
18S rRNA	18 Svedberg Ribosomal Ribonucleic Acid, a component of the 40 Svedberg small eukaryotic ribosomal subunit
28S rRNA	28 Svedberg Ribosomal Ribonucleic Acid, the eukaryotic nuclear homologue of the prokaryotic 23S ribosomal RNA
AHSD	African Horse Sickness Disease
AHSV	African Horse Sickness Virus
AIC	Akaike Information Criterion
AinoV	Aino Virus
BBC	British Broadcasting Corporation
BTD	Bluetongue Disease
BTV	Bluetongue Virus
BTV-1	Bluetongue virus serotype 1
BTV-2	Bluetongue virus serotype 2
BTV-3	Bluetongue virus serotype 3
BTV-4	Bluetongue virus serotype 4
BTV-6	Bluetongue virus serotype 6
BTV-8	Bluetongue virus serotype 8
BTV-9	Bluetongue virus serotype 9
BTV-10	Bluetongue virus serotype 10
BTV-11	Bluetongue virus serotype 11
BTV-16	Bluetongue virus serotype 16
BTV-25	Bluetongue virus serotype 25
BTV-26	Bluetongue virus serotype 26
BTV-27	Bluetongue virus serotype 27
C.	*Culicoides*
CAD	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase, a gene which encodes several enzymes

	involved in pyrimidine biosynthesis
CDC	Centers of Disease Control
CIISA	Centre of Research in Animal Health
cm	Centimeter
CO_2	Carbon Dioxide
COI	Cytochrome Oxidase Subunit I
COII	Cytochrome Oxidase Subunit II
COM	Composed Optical Microscopy
Csa	Hot-summer Mediterranean climate
Csb	Warm-summer Mediterranean climate
Cyt*b*	Cytochrome b
DDT	Dichlorodiphenyltrichloroethane
DEET	*N,N*-Diethyl-3-methylbenzamide
DGV	Direção Geral de Veterinária
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphates
e.g.	*Exempli gratia*, for example
EFSA	European Food Safety Authority
EHD	Epizootic Hemorrhagic Disease
EHDV	Epizootic Hemorrhagic Disease Virus
EID	Entente Interdépartementale pour la Démoustication
ELISA	Enzyme-Linked Immunosorbent Assay
***et al*.**	*et alii*, and others
FCUL	Faculty of Sciences, University of Lisbon
FMV	Faculty of Veterinary Medicine
g/m^2	Gram per square meter
GPS	Global Positioning System
GUs	Geographical Units
h	Hour
HDPE	High-density Polyethylene
ICZN	International Code of Zoological Nomenclature
IDIs	Insect Development Inhibitors

i.e.	*id est*, that is
IGRs	Insect Growth Regulators
IPMA	Instituto Português do Mar e da Atmosfera
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2
JHAs	Juvenile Hormone Analogs
km	Kilometers
km/h	Kilometers per hour
kV	Kilovolt
LC_{99}	Lethal concentration for 99% of a group of test animals
LCS	Liquid crystals
L/W	Length/Width
m	Meter
$MgCl_2$	Magnesium chloride
min	Minute
ML	Maximum Likelihood
ml	Mililiter
MLV	Modified Live Virus
mM	Millimolar
mm	Millimeter
µm	Micrometer
mtDNA	Mitochondrial Deoxyribonucleic Acid
N	North
n	Size of statistical sample
n.d.	Not defined
NESP	National Entomologic Surveillance Program
NUTS III	Nomenclature of Territorial Units for Statistics, subdivision 3
OIE	Office International des Epizooties
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-time Polymerase Chain Reaction
|r|	Pearson correlation coefficient

rDNA	Ribosomal Deoxyribonucleic Acid
ROC	Receiver Operating Curve
S	South
s	Second
SBV	Schmallenberg Virus
SEM	Scanning Electron Microscopy
SM	Stereoscope Microscopy
spp.	Species
syn.	Synonymy
UK	United Kingdom
ULisboa	University of Lisbon
USA	United States of America
UV	Ultraviolet
V	Volt
VBD	Vector-borne Diseases
VP2	Outer capsid viral protein 2
W	Watt
WAHID	World Animal Health Information Database
WHO	World Health Organization
µl	Microliter
µM	Micromolar
µm	Micrometers
°	Degree
°C	Degree Celsius
'	Minute
γ	Gamma
δ	Delta

Chapter 1: General Introduction

1.1. The importance of vector-borne diseases

The morbidity and mortality associated to infectious diseases affects, since ever, the society at the politic, economic and cultural levels (Nelson & Williams, 2007). According to the World Health Organization (WHO, 2014), vector-borne diseases (VBD) account for more than 17% of all infectious diseases, causing more than 1 million deaths annually.

VBD cause huge economic harm when livestock is affected and even the threat of infection can prejudice trade (Lemon *et al*., 2008). The distribution of these diseases depends on complex environmental and social factors such as globalization of travel and trade, unplanned urbanization and environmental challenges. Due to increased human mobility, population growth, trade, climate, ecology and land use changes, some VBD are appearing in countries where they have never been reported before (de Vos, Hoek, Fischer, Koeijer & Bremmer, 2012; WHO, 2014; Faburay, 2015).

An efficient and cost-effective risk management, as well as a deeper insight of the pathways leading to the introduction and spread of VBD, requires an improved knowledge of the mentioned diseases coupled with interdisciplinary collaboration among scientific areas such as epidemiology, virology, entomology, ecology, climatology and economy (de Vos *et al.*, 2012).

Among VBD, those transmitted by arthropods can be referred. There are some kinds of pathogenic agents (*e.g.*, Arboviruses) that need an arthropod vector to complete their life cycle and that are transmitted to the vertebrate host by the arthropod bite or sting (Chippaux, 2003). There are several arthropods that can transmit different kinds of pathogenic agents, and, in this context, biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) can be mentioned (Figure 1.1.).

Figure 1.1. – *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) biting midge.

Culicoides imicola – a female specimen. Scale bar: 100 µm. Original photo.

Culicoides are of major importance in Veterinary Medicine and Public Health since hematophagous females of this genus are known vectors of viruses, filarial nematodes and protozoans, like Bluetongue virus (BTV), Schmallenberg virus (SBV), African Horse Sickness virus (AHSV), Epizootic Haemorrhagic Disease virus (EHDV), *Onchocerca cervicalis, Mansonella* spp., *Haemoproteus meleagridis*, *Leucocytozoon caulleryi*, among others.

Bluetongue Disease (BTD), African Horse Sickness Disease (AHSD) and Epizootic Haemorrhagic Disease (EHD), all classified as notifiable by the Office International des Epizooties (OIE) (Venter, Labuschagne, Boikanyo, Morey & Snyman, 2011), have huge economical impact to producers and affected countries; as example, BTD, a viral disease which affects sheep and cattle, results in annual losses of approximately $3 billion due to morbidity and mortality of affected animals, trade embargoes and vaccination costs (Osburn, 2008; Maclachlan & Mayo, 2013). Table 1.1. shows some viral diseases transmitted by *Culicoides* species of Western Europe.

Moreover, they are a major tourist problem and an occupational concern for people working outdoors due to their nuisance and persistent, painful bite (Mullen & Durden, 2009; de Heredia & Lafuente, 2011; Elbers, Meiswinkel, van Weezep, Sloet van Oldruitenborgh-Oosterbaan, Kooi, 2013). Their bite is also responsible for the Equine Allergic Dermatitis, a severe allergic reaction observed in horses (Mullen & Durden, 2009).

Table 1.1. – Viruses transmitted by *Culicoides* species present in Western Europe.

Virus	**Disease**	**Species affected**	***Culicoides* species from Western Europe**	**References**
AHSV	African Horse Sickness	Horses, mules, donkey, zebra	*C. imicola* Mixed pool *C. obsoletus/C. pulicaris*	Mellor *et al.*, 1990
AinoV	Aino disease	Cattle, sheep	*C. punctatus*	Yanase *et al.*, 2005
AKAV	Akabane disease	Ruminants	*C. imicola* *C. kingi* *C. nubeculosus*[1]	Jennings & Mellor, 1989 Bryant *et al.*, 2005 Fall *et al*., 2015
BTV	Bluetongue disease	Domestic and wild ruminants	*C. achrayi* *C. chiopterus* *C. dewulfi* *C. imicola* *C. lupicaris* *C. montanus* *C. newsteadi* *C. obsoletus* *C. pulicaris* *C. punctatus* *C. scoticus* Nubeculosus complex[2]	Hoffmann *et al.*, 2009 Maclachlan, 2011 Romón *et al.*, 2012 Goffredo *et al*., 2015
EEV	Equine encephalosis	All equine species	*C. imicola*	Gordon *et al.*, 2015

Table 1.1. – Viruses transmitted by *Culicoides* species present in Western Europe (Continuation).

Virus	Disease/Virus name	Species affected	*Culicoides* species from Western Europe	References
EHDV	Epizootic hemorrhagic disease	Domestic and wild ruminants	*C. circumscriptus* *C. festivipennis* *C. gejgelensis* *C. imicola* *C. kingi* *C. longipennis* *C. nubeculosus* *C. obsoletus* *C. pulicaris* *C. punctatus*	Savini *et al.*, 2011 Dik *et al.*, 2012
MDV	Main drain disease	Hares and rabbits	*C. nubeculosus*[1]	Mellor *et al.*, 1974
SBV	Schmallenberg disease	Ruminants	*C. chiopterus* *C. dewulfi* *C. imicola* *C. nubeculosus*[1] *C. obsoletus* *C. pulicaris* *C. punctatus* *C. scoticus*	De Regge *et al.*, 2012 Larska *et al.*, 2013 Balenghien *et al.*, 2014 EFSA, 2014 Zimmer *et al.*, 2015

[1]Biting midges experimentally infected; [2]Includes, at least, *C. nubeculosus*, *C. puncticollis* and *C. riethi*.

Culicoides are distributed worldwide and can be found in a wide range of habitats, from sea level to 4 000 m in altitude, and in almost all countries in the world, with exception of small regions of New Zealand, Patagonia, Hawaii islands, Iceland and Antarctica (Mellor, Boorman & Baylis, 2000; de Heredia & Lafuente, 2011). Planet Earth is divided into several zoogeographical regions, based on distributional patterns of animal organisms, and Europe is included in the Palearctic region (Figure 1.2.) (Procheş & Ramdhani, 2012). Although *Culicoides* species are distributed worldwide, some of them are characteristic of certain Earth regions (Wilson & Mellor, 2009; Mathieu *et al*., 2012) (Annex 1.1.).

Figure 1.2. – Palearctic region (adapted from Procheş & Ramdhani, 2012).

1.2. *Culicoides* taxonomy – a classification far from finished

According to Fauna Europaea website (2015), the taxonomic position of *Culicoides* genus is as follows:

Kingdom	Animalia
Subkingdom	Eumetazoa
Phylum	Arthropoda
Subphylum	Hexapoda
Class	Insecta
Order	Diptera
Suborder	Nematocera
Infraorder	Culicomorpha
Superfamily	Chironomoidea
Family	Ceratopogonidae
Subfamily	Ceratopogoninae
Tribe	Culicoidini
Genus	*Culicoides*

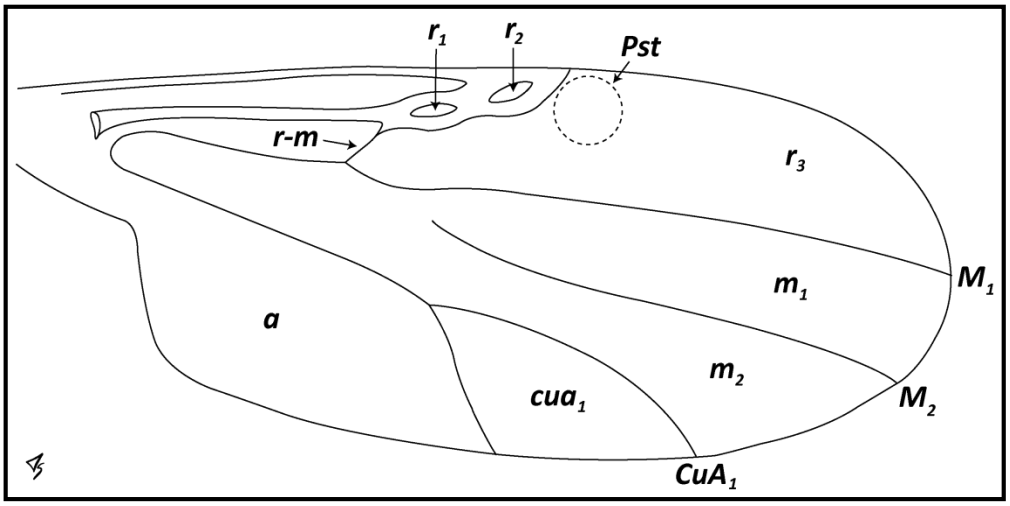
Since the first descriptions of these biting midges by William Derham in 1713 (as *Culex*), Carl von Linné in 1758 (as *Culex* genus) and finally Latreille in 1809 as *Culicoides*, many species inside this genus have been described, some of them being synonyms (Delécolle, 1985; Mathieu, 2011). Up to 2015, *Culicoides* genus was divided into 31 subgenus and 38 species groups (unplaced to subgenus). A total of 1 401 species is identified worldwide (1 355 extant species and 46 fossil species), although a hard systematic work must be done in order to establish some organization inside this genus (Borkent, 2015a).

1.2.1. *Culicoides* genus and species identification

Ceratopogonidae family includes four subfamilies: Dasyheleinae, Leptoconopinae, Forcipomyiinae and Ceratopogoninae (de Heredia & Lafuente, 2011). Ceratopogonids characteristic wing venation allows us to distinguish these midges from other groups of flies (Mullen & Durden, 2009). Midges from Ceratopogonidae family are sometimes very similar between different genus and an accurate evaluation with stereoscope microscopy (SM) must be performed to separate *Culicoides* genus from others (de Heredia & Lafuente, 2011).

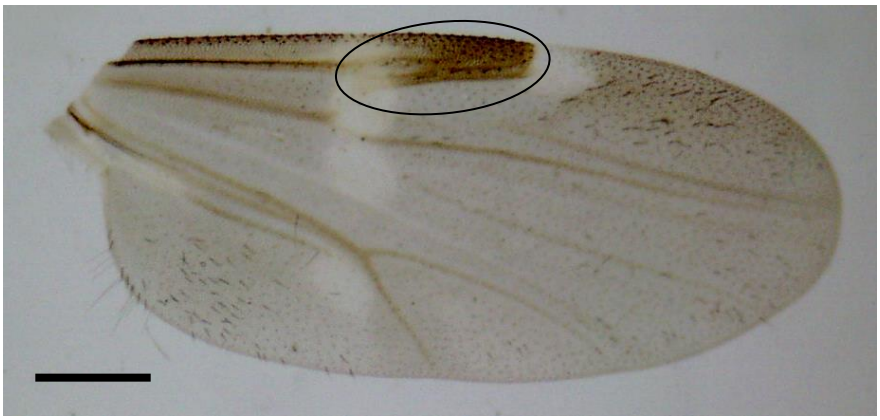
Culicoides biting midges have two open radial cells with similar sizes on the wing (although in some species the second can be bigger than the first) (Figures 1.3. and 1.4.) (Mathieu, 2011). Furthermore, the presence of not aligned spines in the first hind tarsus can also be used to distinguish *Culicoides* specimens from other ceratopogonids (de Heredia & Lafuente, 2011).

Figure 1.3. - Cells and veins of a *Culicoides* wing (scheme by Santos, M.).



r-m – Radio-medial crossvein; r_1 – First radial cell; r_2 – Second radial cell; r_3 – Third radial cell; m_1 – First medial cell; m_2 – Second medial cell; cua_1 – Anterior cubital cell; a – Anal cell; Pst – Poststigmatic pale spot; M_1 – First medial vein; M_2 – Second medial vein; CuA_1 – First branch of anterior cubital vein.

Figure 1.4. – *Culicoides fascipennis* wing.



The two radial cells are inside the dark circle. Scale bar: 200 µm. Original photo.

The wing pattern present in most *Culicoides* species results from the distribution of small and big hairs on wing surface named microtrichia and macrotrichia, respectively. The density of these hairs gives to the wings the appearance of dark and light spots or patches (Figure 1.4.). The shape, disposition or absence of these spots is of most importance, since it is the main characteristic that permits *Culicoides* species identification (Mullen & Durden, 2009; de Heredia & Lafuente, 2011; Mathieu, 2011; Service, 2012). However, it must be taken into account that wing pattern can show high levels of intraspecific variation (Chaker, Delécolle & Kremer, 1980; Wirth, Dyce & Peterson, 1985; Wirth, Dyce & Spinelli, 1988; Felippe-Bauer, Cáceres, Silva, Valderrama-Bazan & Gonzales-Perez, 2005; Felippe-Bauer & Silva, 2006; Felippe-Bauer *et al*., 2008; Felippe-Bauer, Damasceno, da Trindade & Py-Daniel, 2010), giving rise to confusing situations and sometimes rendering difficult to allocate similar wing patterns to either the same or to different species (Figure 1.5.).

Figure 1.5. – *Culicoides circumscriptus* female wings with intraspecific variation.

Intraspecific variation can be observed in the spot inside the dark circle. Scale bars: 200 µm. Original photos.

1.2.2. *Culicoides* subgenera

Some *Culicoides* species are grouped into the same subgenus, for they share some identic morphological characteristics (Borkent, 2015b,c). In Iberian Peninsula, the following *Culicoides* subgenera can be found: *Avaritia* Fox, 1955, *Beltranmyia* Vargas, 1953, *Culicoides* Latreille, 1809, *Monoculicoides* Khalaf, 1954, *Oecacta* Poey, 1853, *Pontoculicoides* Remm, 1968, *Silvaticulicoides* Glukhova, 1977, *Synhelea* Kieffer, 1925 and *Wirthomyia* Vargas, 1973 (Iberfauna, 2008; de Heredia & Lafuente, 2011; Borkent, 2015b).

Almost all *Culicoides* species vectors of BTV and SBV in Europe belong to *Avaritia* subgenus (Wirth & Dyce, 1985; Meiswinkel, Gomulski, Delécolle, Goffredo & Gasperi, 2004a; de Heredia & Lafuente, 2011; Mathieu, 2011). Several species do not have a defined subgenus and are included into the *Oecacta* subgenus, showing, once more, that *Culicoides* systematic classification is far from finished (Jones *et al.,* 1985; de Heredia & Lafuente, 2011). Each subgenus has a type species (Harrup, Bellis, Balenghien & Garros, 2014).

1.2.3. *Culicoides* groups and complexes

Some *Culicoides* species share the same or have similar wing patterns and, in this way, their differentiation is done under evaluation of other morphological characteristics; sometimes, these species are arranged into the same group, for they exhibit a notable degree of morphological differentiation although they are close related species (Mathieu, 2011; Harrup *et al*., 2014).

There are many different *Culicoides* groups mentioned in literature and, from those, Imicola, Obsoletus and Pulicaris groups [defined in the past as complexes (Meiswinkel *et al*., 2004a; Nolan *et al*., 2007; Pagès *et al*., 2009)] can be referred, since they have many *Culicoides*

species proven or incriminated as vectors of several animal and human diseases worldwide (Hoffmann *et al*., 2009; Mathieu, 2011; Rasmussen *et al*., 2012; De Regge *et al.*, 2012; Harrup *et al*., 2014).

'Species complex' is composed by isomorphic species, which are extremely difficult to be morphologically differentiated in one or both sexes (Harrup *et al*., 2014).

As examples:

1) Obsoletus group consists of five species in Western Europe, all of them from *Avaritia* subgenus, with the same wing pattern: *Culicoides obsoletus*, *C. scoticus*, *C. chiopterus*, *C. dewulfi* and *C. montanus* (Figure 1.6.) (Meiswinkel *et al.*, 2004a; Garros, Mathieu, Balenghien, Cêtre-Sossah & Delécolle, 2010; Venail *et al*., 2012; Meiswinkel *et al*., 2014a). Inside this group and after composed optical microscopy (COM) evaluation, *C. chiopterus* and *C. dewulfi* female specimens can be easily distinguished from those of the other three species. These last ones, due to their similar morphologic conformation and very difficult differentiation, even after COM evaluation, are grouped into the Obsoletus complex (Delécolle, 1985; Meiswinkel *et al.*, 2004a; Garros *et al*., 2010; Harrup *et al*., 2014). Concerning males of *C. obsoletus* and *C. scoticus* species, they exhibit sufficient and strong differences on their genitalia which permits their differentiation (Delécolle, 1985; Alexandre-Pires *et al*., 2010; Garros *et al*., 2010). However, males from *C. obsoletus* and *C. montanus* are more difficult to distinguish between them since their genitalia differences are minimal (Mathieu, 2011; Kirkeby & Dominiak, 2014).

Figure 1.6. – Wing pattern of Obsoletus group species.

Scale bar: 200 µm. Original photo.

2) *Culicoides* subgenus is composed by several groups and complexes: Pulicaris and Newsteadi groups and Pulicaris, Newsteadi, Fagineus and Impunctatus complexes, just to mention some (Meiswinkel *et al*., 2004a; Gomulski, Meiswinkel, Delécolle, Goffredo & Gasperi, 2006; EFSA, 2008; Pagès *et al*., 2009; Harrup *et al*., 2014). Several works have shown a high level of variation within species of these groups and complexes (Meiswinkel *et al*., 2004a; Pagès *et al*., 2009), being also a subject far from finished. Both Obsoletus and Pulicaris groups are ubiquitous across the Palearctic region (Wilson & Mellor, 2009).

However, some scientific teams do not agree with the inclusion of *C. chiopterus* and *C. dewulfi* into Obsoletus group and, according to them, both should be considered as two independent species; in this way, Obsoletus group is the same as Obsoletus complex, including *C. obsoletus* and *C. scoticus* only (Nolan *et al*., 2007; Schwenkenbecher, Mordue & Piertney, 2009; Hajd Henni, Sauvage, Ninio, Depaquit & Augot, 2014). In this thesis, the classification considered by Garros *et al*. (2014) and Harrup *et al*. (2014) was followed.

Researchers elsewhere found other species related to *C. obsoletus* in Western Europe, like an unidentified pale-winged *Culicoides* species (Gomulski, Meiswinkel, Delécolle, Goffredo & Gaspari, 2005), specimens of the Obsoletus complex which are not molecularly identifiable to species (Sanders *et al*., 2012; Harrup, Purse, Golding, Mellor & Carpenter, 2013), an unknown species of the Obsoletus complex that Wenk, Kaufmann, Schaffner & Mathis (2012) referred to as "*C. obsoletus* O2" and a "dark *obsoletus*" species referred by Meiswinkel, de Bree, Bossers-De Vries & Elbers (2014b) which forms a separated phylogenetic branch from those of *C. obsoletus* and *C. scoticus*, having 90-91% and 87-88% of homology with these last two species, respectively.

It must be referred that this type of classification into 'species group' or 'species complex' is not standardized by the International Code of Zoological Nomenclature (ICZN) and, somehow, it does not alleviate the problem concerning the correct identification of species inside *Culicoides* genus (Harrup *et al*., 2014).

1.2.4. *Culicoides* species in Portugal – a historical review (1952-2005)

The first reference to *Culicoides* genus in Portugal date from 1952 (Cambournac, 1956). Since then, several *Culicoides* studies have been performed in our country and, until 2005, 47 *Culicoides* species had been reported in Portugal (Cambournac, 1970a,b; Mellor, Jennings, Wilkinson & Boorman, 1985; Capela, Kremer, Messaddeq, Lemblé & Waller, 1990; Lamblé, Messaddeq, Capela & Kremer, 1990; Capela, Pena & Kremer, 1992; Capela, Sousa, Pena & Caeiro, 1993; Capela, Pena & Kremer, 1997; Capela *et al*., 2003; Pena, 2003; Diaz, Vieira & Báez, 2005; Vila-Viçosa, Simões & Caeiro, 2009). All species reported from the 1952-2005 period in Portugal (mainland Portugal and Azores and Madeira archipelagos) are referred in Table 1.2. and Table 1.3.. A list of synonymies is also referred in Annex 1.2.

Table 1.2. – *Culicoides* species referred in mainland Portugal (1952-2005).

***Culicoides* species**	**Place/Region of collection**[1]	**References**
C. sintrensis	Sintra, East part of Ribatejo, Torres Vedras	Cambournac, 1956
C. almeidae	Benavente, Coruche, Santo Estêvão, Torres Vedras, A-dos-Cunhados, Vimieiro, Alcácer do Sal, Grândola	Cambournac, 1970a

Table 1.2. – *Culicoides* species referred in mainland Portugal (1952-2005) (Continuation).

***Culicoides* species**	**Place/Region of collection**[1]	**References**
C. brunnicans	Benavente, Coruche, Santo Estêvão	Cambournac, 1970b
C. circumscriptus	East region of Alentejo, Alcácer do Sal, Grândola	
C. fascipennis	Alentejo region, Benavente, Coruche, Santo Estêvão	
C. festivipennis	Alentejo region	
C. impunctatus	East region of Alentejo	
C. nubeculosus	East region of Alentejo	
C. nuntius[2]	Vale d'Arquinha (Torrão region)	
C, obsoletus	East region of Alentejo	
C. parroti	East region of Alentejo	
C. pictipennis	Alentejo region	
C. pulicaris	East region of Alentejo	
C. puncticollis	East region of Alentejo	
C. riethi	East region of Alentejo	
C. rochenus[2]	Sado river valley, from Águas de Moura to Alcácer do Sal	
C. cataneii	Coastal regions between Oporto and Lisbon. *C. imicola* was captured in Pegões	Mellor *et al*., 1985
C. fagineus		
C. gejgelensis		
C. heteroclitus		
C. imicola		
C. jumineri		
C. kibunensis		
C. maritimus		
C. odiatus		
C. punctatus		
C. albihalteratus	Mogadouro	Capela *et al*., 1990
C. atripennis	Aldeia Nova	
C. begueti	Mogadouro	
C. furcillatus	Herdade do Pinheiro	
C. haranti	Elvas	
C. indistinctus	Aldeia Nova, Bragança, Mogadouro	
C. kurensis	Aldeia Nova, Bragança	
C. pallidicornis	Aldeia Nova	
C. pseudopallidus	Pegões	
C. scoticus	Aldeia Nova	
C. sahariensis	Barrancos	
C. subfasciipennis	Aldeia Nova (Montalegre), Bragança	
C. univittatus	Almada do Ouro, Várzea de Sintra	
C. tbilisicus	Aldeia Nova (Trás-os-Montes)	Lamblé *et al*., 1990
C. achrayi	Cheires (Alijó), Pópulo (Vila Chã), Carvalho, Vila Pouca de Aguiar, Freixo de Espada à Cinta	Capela *et al*., 1992
C. chiopterus	Aldeia Nova (Montalegre)	
C. corsicus	Barrancos-Amareleja, Minas de Aparis, Minas de São Domingos, Figueira de Castelo Rodrigo-Pinhel	
C. derisor	Herdade das Mercês	
C. vexans	Freixo de Espada à Cinta	

Table 1.2. – *Culicoides* species referred in mainland Portugal (1952-2005) (Continuation).

***Culicoides* species**	**Place/Region of collection**[1]	**References**
C. shaklawensis	Mercês, Vila Nova de Milfontes	Pena, 2003

[1]First place/region of mainland Portugal where *Culicoides* species were captured; [2]Species that were never captured since their first description.

Table 1.3. – *Culicoides* species referred in Madeira and Azores archipelagos (1990-2005).

***Culicoides* species**	**Portugal region**	**References**
C. obsoletus *C. scoticus*	Madeira archipelago (Madeira and Porto Santo islands)	Capela *et al*., 1990, 1997
C. newsteadi *C. puncticollis*	Madeira archipelago (Porto Santo island)	Capela *et al*., 1990
C. obsoletus	Azores archipelago (S. Miguel, Terceira, S. Jorge, Pico and Faial islands)	Diaz *et al*., 2005

Some situations must be pointed out concerning synonymies and classification of some specimens (Table 1.2. and Annex 1.2.): *C. sintrensis* (species collected in 1952 and reported for the first time in 1956 by Cambournac) is a synonym for *C. obsoletus* (Pena, 2003). *C. nuntius* and *C. rochenus* were never captured again since their brief description by Cambournac (1970b); furthermore, there are no photographs or specimens deposited for comparison (Pena, 2003). Finally, according to Pena (2003), *C. almeidae* can be a synonym of *C. punctatus* or *C. pulicaris*.

1.3. *Culicoides* and Bluetongue disease – a double-connected problem

1.3.1. Brief historical review of Bluetongue disease

Bluetongue disease was noticed for the first time in Cape of Good Hope by the French biologist Francois de Vaillant, between 1781 and 1784 (Gutsche, 1979). Almost one hundred years later, in 1876, the disease was again reported in South Africa by Henning due to introduction of various susceptible sheep breeds from Europe (Hutcheon, 1881, 1902; Howell & Verwoerd, 1971; Erasmus, 1990; de Heredia & Lafuente, 2011).

Bluetongue disease occurred sporadically in Europe, being exotic to this continent, with the exception of Cyprus island, where it occurred several times since 1924 (Polydorou, 1978). In 1979-80, BTD reached several Greek islands (Vassalos, 1980; Dragonas, 1981; Ortega, Mellor, Rawlings & Pro, 1998; de Heredia & Lafuente, 2011). In 1998, BTD appeared in Greece and in 1999 the disease expanded to Turkey and Bulgaria. In 2000, BTD appeared in Tunisia, Argelia, Marrocco, Italy, Corsica and Mallorca and Minorca islands. Between 2003 and 2005, the disease emerged in Portugal, Spain and Corsica island. Finally, the disease appeared in Central Europe (Germany, France, Belgium and Netherlands) in 2006 and in Northern European countries (United Kingdom, Denmark, Sweden and Norway) in 2007-08 (Van Wuijckhuise *et al*., 2006; Elbers *et al*., 2008; Saegerman, Berkvens & Mellor, 2008; Mellor, Baylis & Mertens, 2009; Wilson & Mellor, 2009; Sternberg Lewerin *et al*., 2010).

1.3.2. Bluetongue disease

1.3.2.1. Etiology

Bluetongue is a non-zoonotic disease caused by BTV, the type species of the genus *Orbivirus*, family *Reoviridae*, and is transmitted by *Culicoides* biting midges, affecting domestic and wild ruminants (Hutcheon, 1902; Spreull, 1905; Henning, 1956; Verwoerd & Erasmus, 2004; Mellor *et al*., 2009; Verwoerd, 2012). Although rare, transmission by other vectors (*e.g.*, *Melophagus ovinus* and *Ornithodoros savignyi*) (Luedke, Jochim & Bowne, 1965; Gerdes, 2004; Bouwknegt *et al*., 2010) through bites and skin wounds (López-Olvera *et al*., 2010), oral and vertical transmission (De Clercq *et al*., 2008; Backx, Heutink, van Rooij & van Rijn, 2009) can also occur.

There are, at least, 27 BTV serotypes (BTV-1 to BTV-27) circulating worldwide, being the serotypes determined primarily by differences in VP2, one of the outer capsid proteins of the virus, which induces neutralizing antibodies in infected animals (Huismans & Erasmus, 1981; Kahlon, Sugiyama & Roy, 1983; Mann *et al*., 2007; Hofmann *et al*., 2008; Maan *et al*., 2011; Shaw *et al*., 2013; Jenckel *et al*., 2015).

Before 1998, BTV outbreaks that occurred in Europe tended to be localised, caused by a single serotype and usually limited to a few years duration (Nomikou *et al*., 2015). Those outbreaks included BTV-3, BTV-4 and BTV-10 in countries from the Mediterranean basin, including Iberian Peninsula (Mellor *et al*., 2009). However, between 1998 and 2006 several BTV serotypes have been identified in Europe, especially throughout the Mediterranean coast: BTV-1, BTV-2, BTV-4, BTV-9, BTV-16 (Mellor, Carpenter & Harrup, 2008; Rodríguez-Sánchez, Iglesias-Martín, Martínez-Avilés & Sánchez-Vizcaíno, 2008). In 2006, BTV-8 emerged unexpectedly in northern Europe (Toussaint *et al*., 2006). In 2008, two more BTV serotypes were detected in northern Europe: BTV-6 and BTV-11 (De Clercq *et al*., 2009).

Recently, BTV-25 and BTV-27 serotypes have been detected in goats from Switzerland and Corsica island, respectively (Hofmann *et al*., 2008, Jenckel *et al*., 2015).

1.3.2.2. Affected species and pathogeny

In general, sheep, yaks, llamas, alpacas and white-tailed deer have been described as the most sensitive species to BTV-induced disease, while cattle and other wild ruminants have a certain degree of resistance to BTD, although they are fully susceptible to infection (Maclachlan & Mayo, 2013; Caporale, 2014). As cattle show longer periods of viremia, they are considered reservoirs of infection (Barratt-Boyes & Maclachlan, 1994, 1995; Darpel *et al*., 2007; Henrich, Reinacher & Hamann, 2007; Mauroy *et al*., 2008; Maclachlan, Drew, Darpel & Worwa, 2009; Meyer *et al*., 2009; Falconi, López-Olvera & Gortazar, 2011). Goats are also susceptible to the infection by BTV but they are not very susceptible to the disease (Spreull, 1905; Luedke & Anakwenze, 1972; Erasmus, 1975; Koumbati, Mangana, Nomikou, Mellor &

Papadopoulos, 1999; Backx, Heutink, van Rooij & van Rijn, 2007; Dercksen *et al*., 2007; Coetzee *et al.,* 2013).

The major lesions observed in affected animals are related to the injury of small blood vessels, increasing vascular permeability by capillary leakage. BTV has also tropism for dendritic cells, endothelium and mononuclear leukocytes. The virus can cause thrombocytopenia, coagulopathy and haemorrhagic diathesis (DeMaula, Jutila, Wilson & Maclachlan, 2001; DeMaula, Leutenegger, Bonneau & Maclachlan, 2002; Gowen & Holbrook, 2008; Hemati *et al*., 2009; Maclachlan *et al*., 2009; Drew *et al*., 2010a; Drew, Heller, Mayo, Watson & Maclachlan, 2010b; Lee, Chen, Lin & Wang, 2011; Channappavar *et al*., 2012).

BTV infection in ruminants is characterized by a highly cell-associated viremia and, in the later stages of the disease, it is associated mainly with erythrocytes (Luedke, Jochim & Jones, 1969; Maclachlan, Jagels, Rossitto, Moore & Heidner, 1990; Afshar, 1994; Barratt-Boyes & Maclachlan, 1994; Maclachlan *et al*., 2009), protecting the virus from immune clearance and resulting in a mechanism for infection of *Culicoides* midges when they perform their blood meal (Brewer & Maclachlan, 1992; Maclachlan *et al*., 1994).

1.3.2.3. Clinical signs

There are variable clinical outcomes as a result of BTV infection. In many cases, BTV induces mild or unapparent clinical infections, especially in endemic areas, in cattle and wild African ungulates, while in other cases the infected host dies (Maclachlan & Mayo, 2013; Caporale, 2014).

The main clinical signs of affected animals include high fever, respiratory distress (due to pulmonary edema with accompanying pleural and pericardial effusion in fatal cases), depression and anorexia. Other clinical sings are hyperaemia, congestion, vascular thrombosis, localized or diffused edemas (due to capillary leakage), haemorrhages, erosion of the mucous membranes, ulceration of the oral and nasal mucosa and of the mucosal lining of the upper gastrointestinal tract, coronitis with consequent lameness and multicentric necrosis of both skeletal and cardiac muscle (Spreull, 1905; Moulton, 1961; Howerth, Greene & Prestwood, 1988; Verwoerd & Erasmus, 2004; Backx *et al*., 2007; Mehlhorn *et al*., 2007; Maclachlan & Osburn, 2008; Maclachlan *et al*., 2008, 2009; Maclachlan & Mayo, 2013). BTV-8 serotype can also cause abortions and the birth of non-viable offspring in cattle (Desmecht *et al*., 2008).

Environmental factors, such as solar radiation exposure or high temperatures, can also exacerbate the disease symptoms (Neitz & Riemerschmid, 1944; Verwoerd & Erasmus, 2004).

1.3.2.4. Control

In a first approach, stabling of livestock can prevent the animals against vector attack, when talking about species which rest outside human-made structures (exophilic species). This is the main procedure with horses in South Africa, since their stabling, together with insect-proof quarantine or universally effective insect repellent, highly reduces *C. imicola* bite, a mainly exophilic species (Barnard, 1997; Meiswinkel, Baylis & Labuschagne, 2000; Venail *et al*., 2015).

According to Baylis *et al*. (2010), species belonging to Obsoletus group are also exophilic regardless if animals are inside or outside the stables. However, the endophilic/exophilic activity exhibited by the vector species resident in each area varies substantially (Viennet *et al*., 2012; Maclachlan & Mayo, 2013).

The reactive strategies to control BTD outbreaks or unanticipated incursions of BTV rely on rapid detection of either virus (virological surveillance) or disease (clinical surveillance). This surveillance must include the evaluation of sentinel livestock and, ideally, surveillance of the potential vector midge species present nearby (Maclachlan & Mayo, 2013). Virological diagnosis must be performed with appropriate sensitive and specific assays. Similarly, vector surveillance must include the most correct methods for trapping midges, which may vary depending on *Culicoides* species of interest (Souza Monteiro, Carrasco, Moffitt & Cook, 2012), being the use of black-light traps the most preferred method. Other control measures that can be undertaken to control *Culicoides* midges are described in more detail in 1.8..

Virological diagnosis relies nowadays on specifically group-reactive quantitative real-time polymerase chain reaction (qPCR) assays that reliably detect all BTV serotypes and strains with high levels of sensibility and specificity (Hofmann *et al*., 2008; Mayo *et al*., 2010, 2012a,b; van Rijn, Heutink, Boonstra, Kramps & van Gennip, 2012). Serotype-specific qPCR assays can be also used for rapid and specific determination of virus serotype, without the requirement for expensive and time-consuming virus isolation (Maan *et al*, 2012; Mayo *et al*., 2012b; Maclachlan *et al*., 2013). Quantitative PCR assays are considerably more sensitive than conventional virus isolation and qPCR can also detect viral nucleic acid long after virus cannot be isolated from the blood and tissues of exposed animals.

Highly sensitive and specific competitive enzyme-linked immunosorbent assays (ELISA) are available for serological detection of BTV infection of livestock (Maclachlan & Mayo, 2013). An indirect ELISA for detection of BTV-specific antibodies in bovine milk samples is also available (Kramps, van Maanen, Mars, Popma & van Rijn, 2008).

Vaccination is the most important measure to take in most at-risk countries to any BTD outbreak (Maclachlan & Mayo, 2013). However, vaccination can be problematic due to the plurality of BTV serotypes, together with apparent serotype-specific immunity on livestock (Noad & Roy, 2009; Oya Alpar *et al*., 2009; Zientara, MacLachlan, Calistri, Sanchez-Vizcaino, Savini, 2010). Furthermore, live and attenuated (modified live virus [MLV])

vaccines, which are routinely used in sheep and cattle from different countries, can be acquired and transmitted by insect vectors and, then, circulate as field strains. From here they can reassert gene segments with field viruses to generate novel progeny (Osburn, de Mattos, de Mattos & MacLachlan, 1996; Ferrari *et al*., 2005; Batten, Maan, Shaw, Maan & Mertens, 2008). Finally, MLV vaccines can cross the placenta and infect the fetus (Schultz & Delay, 1955; Flanagan & Johnson, 1995; MacLachlan, Conley & Kennedy, 2000; Maclachlan & Osburn, 2008, Savini *et al*., 2014).

Inactivated BTV vaccines cannot revert virulence, reassort genes with field or MLV viruses or cross the placenta, causing reproductive losses, being advantageous over MLV vaccines. However, they produce a relative slow onset of immunity, requiring booster immunizations when compared with MLV vaccines, have high costs of production, as vaccination requires large amounts of antigen, and there is a lack of commercial products for most serotypes (Savini, MacLachlan, Sanchez-Vizcaino & Zientara, 2008; Maclachlan & Mayo, 2013).

1.3.3. *Culicoides* as vectors of Bluetongue disease

Insects were incriminated as vectors of BTD in 1902 by Hutcheon and Spreull (Hutcheon, 1902; Spreull, 1905). However, it was not until 42 years later, after exhaustive scientific studies, that *Culicoides* biting midges (more specifically *C. imicola*) were pointed out as vectors of this disease (Du Toit, 1944).

Being BTD typical in tropical and temperate climates, distributed between 35°S and 40°N parallels, the sporadic occurrences of BTD in Europe were attributed to *C. imicola* species, the main vector in African continent and that was also present in South European countries (Walton, 2004; Wilson & Mellor, 2009). However, BTV had already been isolated in small laboratory studies from species belonging to Pulicaris and Obsoletus groups, all of them present in European continent (Mellor & Pitzolis, 1979; Mellor, 1990). Although these studies were performed, some scientific communities defended that the probability for those species to acquire, maintain and spread the virus was low, since the disease had never spread to Central and Northern European countries before (Gibbs & Greiner, 1994; Walton, 2004; Carpenter, Wilson & Mellor, 2009). Besides, climatic barriers and those concerning geographical movement of hosts and vectors to Europe were enough to prevent the breakthrough of the disease in North zones of the European continent (Gibbs & Greiner, 1994). However, this paradigm was called into question when the disease appeared in Kosovo in 2001, a place where *C. imicola* was absent (Wilson & Mellor, 2009). Although some doubts still existed between 2001 and 2005, BTD appeared until 44°55'N of the European continent, affecting various countries (Mellor *et al*., 2009).

However, the unexpected occurred in 2006, when the disease spread from Central Europe (where *C. imicola* was definitely absent), probably due to multiple factors, such as global warming, introduction of hosts or infected products from endemic regions or by introduction

of infected vectors, with the disease being detected as far as the 58°N parallel in the subsequent years (Sellers & Taylor, 1980; Sellers, 1980; Carpenter *et al*., 2009; Verwoerd, 2009; Wilson & Mellor, 2009; Sternberg Lewerin *et al*., 2010).

To date, in Europe, BTV was isolated from *C. imicola*, *C. obsoletus*/*C. scoticus* and *C. pulicaris*. The genome of this virus was also detected in parous females (with a complete reproductive cycle) of *C. dewulfi*, *C. chiopterus*, *C. lupicaris*, *C. obsoletus* (Caracappa *et al*., 2003; Savini *et al*., 2005, Vanbinst *et al*., 2009; Romón *et al*., 2012), *C. newsteadi*, *C. punctatus*, *C. montanus* and species belonging to Nubeculosus complex (Goffredo *et al*., 2015).

1.3.4. Bluetongue disease in mainland Portugal

The first incursion of BTV in Portugal was reported in July 1956, affecting mainly sheep flocks in the southern region of the country below Tagus River, excluding Algarve region, and caused by a strain of BTV-10 (Barros *et al*., 2007; Ribeiro *et al*., 2015). The number of outbreaks markedly decreased soon after a monovalent live-attenuated vaccine was used to vaccinate sheep in the affected area of the country and in 1958 the disease practically disappeared. The country was declared free of BTV in 1960 (Barros *et al*., 2007). Between 1956-60, BTV killed more than 179.000 sheep (Manso-Ribeiro *et al*., 1957; López & Botija, 1958).

A new introduction of BTV (BTV-4) in mainland Portugal was registered on the 24th November 2004, after a 44 year period of epizootic silence, in areas of the central-west and southern regions of Portugal bordering Spain. This resulted in an outbreak which persisted until the end of 2006 (OIE, n.d.; Barros *et al*., 2007; Rodrigues, 2008; Ribeiro *et al*., 2015).

In September 2007, a strain of BTV-1 was detected in an Alentejo county located at the Spanish border, two months after a BTV-1 outbreak in Spain (DGAV, 2012). Between September and December 2007, 158 BTV-1 outbreaks were reported in regions in the south and center of the country. However, since 2008, BTV-1 has spread to the north of the country showing a different distribution than BTV-4, BTV-10 and AHSD, occurring 78 BTV-1 outbreaks in the center and south (83%) and in the northern (17%) regions. In 2009, the majority of the 129 BTV-1 outbreaks were reported in the northern regions (67%) and in 2010 only six BTV-1 outbreaks occurred in the center and southern regions (Ribeiro *et al*., 2015). Plus, in 2011 and 2012, one and three BTV-1 outbreaks, respectively, were confirmed (Ribeiro *et al*., 2015). The vaccination campaigns against BTV-4 (2005-2008) and BTV-1 (2007-2010) in sheep and cattle have contributed for the control of the disease.

In 2013, BTV-4 re-emerged again in the mainland territory, causing 10 outbreaks in Algarve region (European Commission, 2012; DGAV, 2012, 2014). These outbreaks determined the adoption of control measures including a mandatory and an optional vaccination of sheep

and cattle, respectively, in Algarve region against BTV-4. Also, it was decided to allow vaccination as a prophylactic measure against BTV-4 in Alentejo region (DGAV, 2015).
Finally, in September 2015, after an epizootic silence of three years, BTV-1 was detected again in Alentejo region, in the councils of Serpa, Moura and Barrancos, among other occurrences that are under investigation in Alentejo and Algarve regions (DGAV, 2015).
At the present time, BTV-1 is circulating in all mainland Portugal, while BTV-4 is circulating only in Algarve region. Voluntary vaccination of sheep and cattle present in the totality of mainland Portugal is allowed, while vaccination of the existent sheep in Castelo Branco, Idanha-a-Nova and Vila Velha de Rodão councils is mandatory. As 22nd May, 2015, Azores and Madeira archipelagos were constituted as BTD free zones. Councils from North, Center and Lisboa and Vale do Tejo geographic areas are subjected to animal movement restrictions due to BTV-1. The geographic areas of Alentejo and Algarve are subjected to the same restrictions due to BTV-1 and BTV-4, although in Alentejo this last serotype is considered as low risk of viral circulation (DGAV, 2015).

1.4. African Horse Sickness disease in mainland Portugal

African Horse Sickness was first detected in Casto Marim, Portugal, in August 1989, near the Spanish frontier (Pena, 2003). The disease spread throughout the Alentejo region (Barrancos), along the Portuguese border.
Several control measures were taken: entomological studies concerning *Culicoides* midges, massive vaccination of horses, donkeys and mules, insecticide aspersion of the horse stables and, finally, distribution of adequate information to the owners (*e.g.*, stabling of animals during night, isolation of sick animals), with the final aim to prevent the spread of the disease outside the affected area (Pena, 2003).
In 1991, no cases of the disease were detected in Portugal. Until the end of 1989, the disease killed 202 horses in the Guadiana river zone. In 1992, Portugal was considered free from the disease (Pena, 2003).

1.5. Adult *Culicoides* morphology

Adult *Culicoides* midges are small insects (from 0.5 to 3 mm) composed by three body parts: head, thorax and abdomen (Figure 1.7.). In these body parts, different structures, which are extremely important for *Culicoides* species identification when wing pattern is equal or very similar between them, can be found (Mathieu, 2011; Garros *et al.*, 2014; Hajd Henni, de Meulemeester, Mathieu, Depaquit & Augot, 2015).

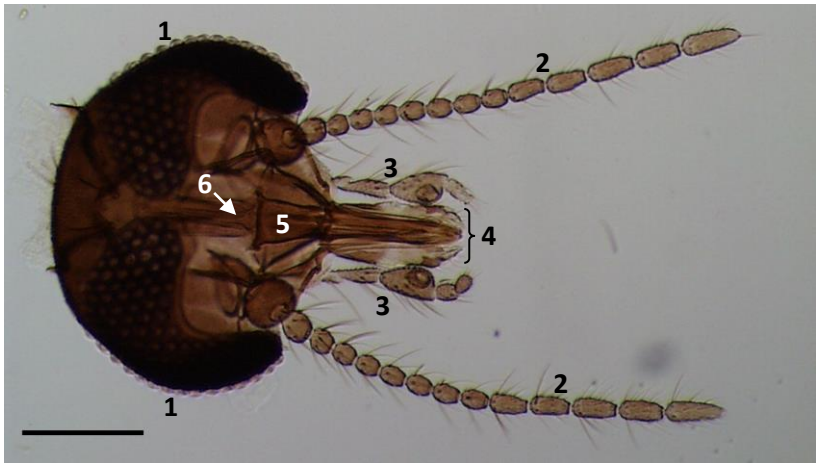
Figure 1.7. – *Culicoides* female specimen from Obsoletus group.

1 – Head; 2 – Thorax; 3 – Abdomen. Scale bar: 100 µm. Original photo.

1.5.1. Head

The following anatomical structures are present in the head: eyes, antennas, palpi, mouth parts, cibarium and posterior pharynx (Figure 1.8.).

Figure 1.8. – *Culicoides derisor* female's head.

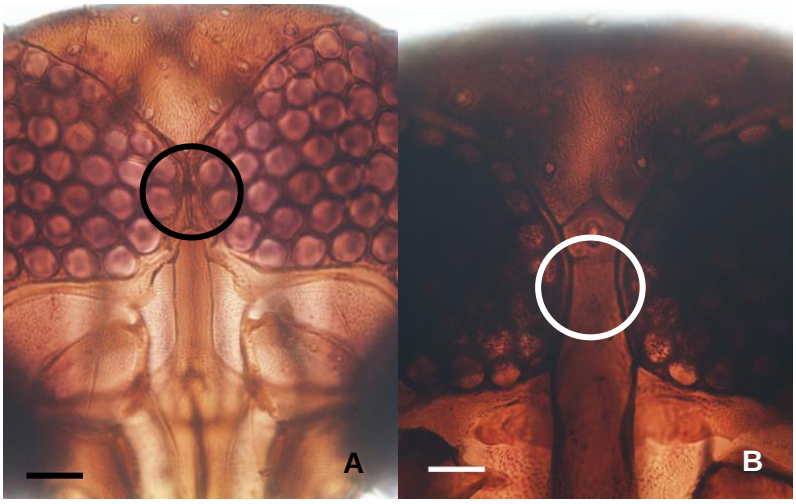


1 – Eyes; 2 – Antennas; 3 – Palpi; 4 – Mouth parts; 5 – Cibarium;
6 – Posterior pharynx. Scale bar: 100 µm. Original photo.

1.5.1.1. Eyes

Culicoides biting midges have eyes constituted by ommatidia or facets. Ommatidia are composed of both optical and sensorial parts (Maggenti, Maggenti & Gardner, 2005). In females, the eyes can be joined (holoptic) or separated (dichoptic), depending on species (Figure 1.9.). Some species have pubescence between the ommatidia. Males are always holoptic. Ocelli are absent in *Culicoides* genus (de Heredia & Lafuente, 2011; Mathieu, 2011). Finally, all *Culicoides* species possess interocular hairs (Downes & Wirth, 1981; Borkent, 2004).

Figure 1.9. – Holoptic and dichoptic species.



A – *Culicoides imicola*: a species with joined eyes (holoptic) (black circle); B – *Culicoides circumscriptus*: a species with separated eyes (dichoptic) (white circle). Scale bars: 20 µm. Original photos.

1.5.1.2. Antennas

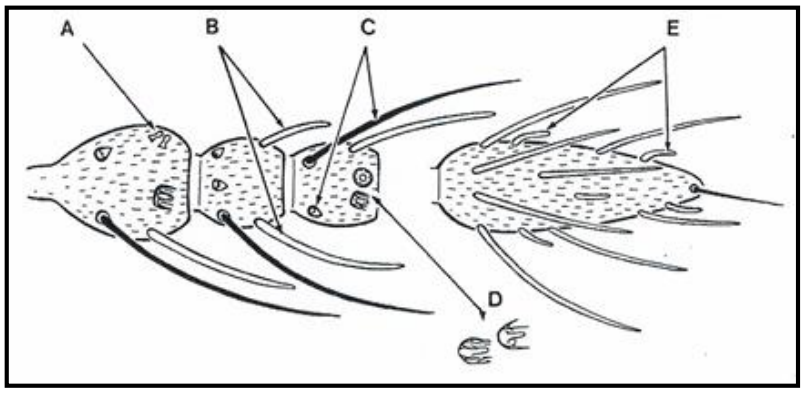
Culicoides antennas are composed by three parts: the basal, ring-shaped scape, a large pedicel and the flagellum, which is secondarily segmented into 13 flagellomeres (Swanson, 2012).

In females, from the 1st to the 8th flagellomere they are generally called short flagellomeres and from the 9th to the 13th flagellomere they are called long flagellomeres. In some literature, short flagellomeres are numbered from III to X and long flagellomeres from XI to XV, since the entire antenna is composed by 15 segments (Delécolle, 1985; Mathieu *et al*., 2012).

In each flagellomere different kinds of sensorial organs, called sensilla, can be found. These sensorial organs have different conformations and they are named differently according to their morphological structure (Maggenti *et al*., 2005) (Figure 1.10.):

a) sensillum ampullaceum (with a flask- or pouch-shaped cavity).
b) sensillum trichodeum (a sense organ bearing an elongate hair, which can be short or long).
c) sensillum chaeticum (an external process with spine- or bristle-like form, being tactile in function).
d) sensillum basiconicum (an external process in the form of a minute cone or peg).
e) sensillum coeloconicum (an external process in the form of a thin-walled conical or peg-like projection in a shallow pit below the surface of the body wall).

Figure 1.10. – Different kinds of sensilla found in *Culicoides* genus antenna (adapted from Delécolle, 1985).



A – Sensilla ampullacea; B – Sensilla trichodea (short and long); C – Sensilla chaetica; D – Sensillum coeloconica; E – Sensilla basiconica.

Sensilla have several physiologic functions in different kinds of insects (Davis & Sokolove, 1975; Wirth & Navai, 1978; Barlin, Vinson & Piper, 1981; Chapman, 1982; Blackwell, Mordue & Mordue, 1992a; Olsen & Andow, 1993; Bowen, 1995; Isidoro, Bin, Colazza & Vinson, 1996; van Baaren, Barbier & Nénon, 1996; Amornsak, Cribb & Gordh, 1998; van Baaren, Boivin, Lannic & Nénon, 1999; Kleineidam, Romani, Tautz & Isidoro, 2000; Pettersson, Hallberg & Biggersson, 2001; Bleeker, Smid, van Aelst, van Loon & Vet, 2004; Maggenti *et al*., 2005; Roux, van Baaren, Gers, Arvanitakis & Legal, 2005; Marques-Silva *et al*., 2006; Onagbola & Fadamiro, 2008):

a) Sensillum ampullaceum is responsible for carbon dioxide (CO_2) perception in *Atta sexdens* (Order Hymenoptera, Family Formicidae).

b) Sensillum trichodeum is divided into several types in some insect groups (*e.g.*, Hymenoptera) and is likely to have mechanoreceptive functions, such as in the perception of mechanosensory stimuli, in host examination and discrimination, detection of sex pheromones or as proprioceptors.

c) Sensillum basiconicum is composed by short and long grooved pegs in some group of insects (e.g., *Aedes* and *Culex* genus), being the short grooved pegs sensible to lactic acid and both sensible to butyric acid in *Aedes* genus.

d) Sensillum chaeticum functions as mechanoreceptor or as mechanosensorial receptor.

e) Sensillum coeloconicum can act as thermoreceptor, responding to temperature changes, as it happen in mosquitoes. Furthermore, this sensillum responds to CO_2 and humidity. Sensillum coeloconicum is of extreme importance in *Culicoides* species identification, since its presence in antennal flagellomeres is variable between different species (de Heredia & Lafuente, 2011; Mathieu *et al*., 2012). Female antenna is hairy.

In males, although the same structures are observed, there are some different aspects from females: the pedicel is bigger, the short flagellomeres occur from the 1st to the 10th (III-XII)

flagellomeres and the long flagellomeres from the 11th to the 13th (XIII-XV) flagellomeres. Male antenna is plumy (de Heredia & Lafuente, 2011; Mathieu, 2011).

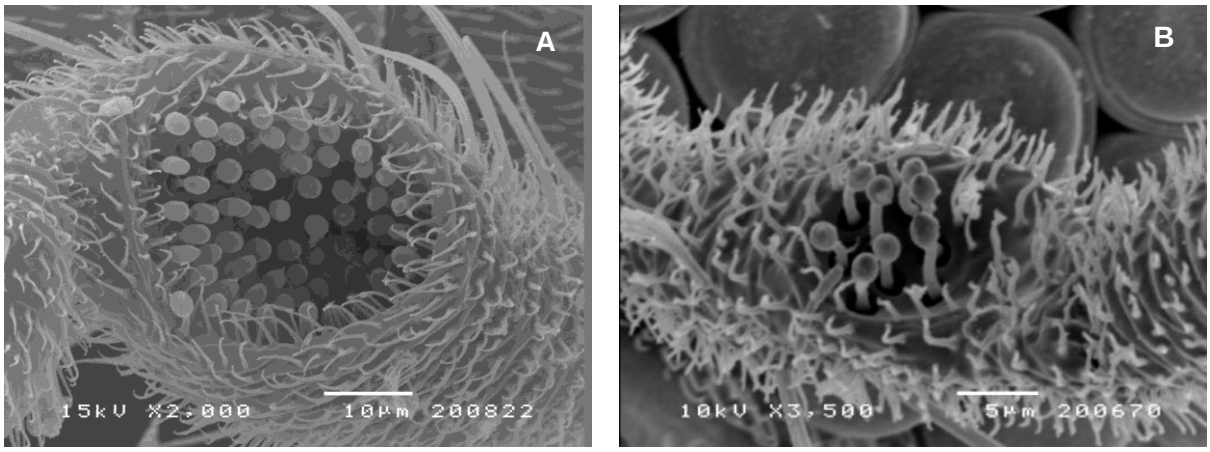
1.5.1.3. Palpi

Palpi or maxillary palp are structures arising from both sides of mouth parts. In both sexes, palpi are composed of five segments, being the first and the second fused.

The 3rd palpus segment has a sensorial organ called sensorial pit, which is composed by several sensilla basiconica with a bulb-shaped form (Alexandre-Pires *et al*., 2010; de Heredia & Lafuente, 2011; Isberg, Hillbur & Ignell, 2013).

Females have a higher number of sensilla basiconica than males, since these sensorial organs are specialized in detecting CO_2, feature that hematophagous females use to detect hosts, while males are only phytophagous and do not need them in a large number (Rowley & Cornford, 1972; Grant & Kline, 2003; de Heredia & Lafuente, 2011; Mathieu, 2011) (Figure 1.11.).

Figure 1.11. – Sensilla basiconica within the sensorial pit of the 3rd palpus segment of a female (A) and a male (B) specimen (Alexandre-Pires *et al.,* 2010).



A – *Culicoides circumscriptus* female specimen; B – *Culicoides obsoletus* male specimen.

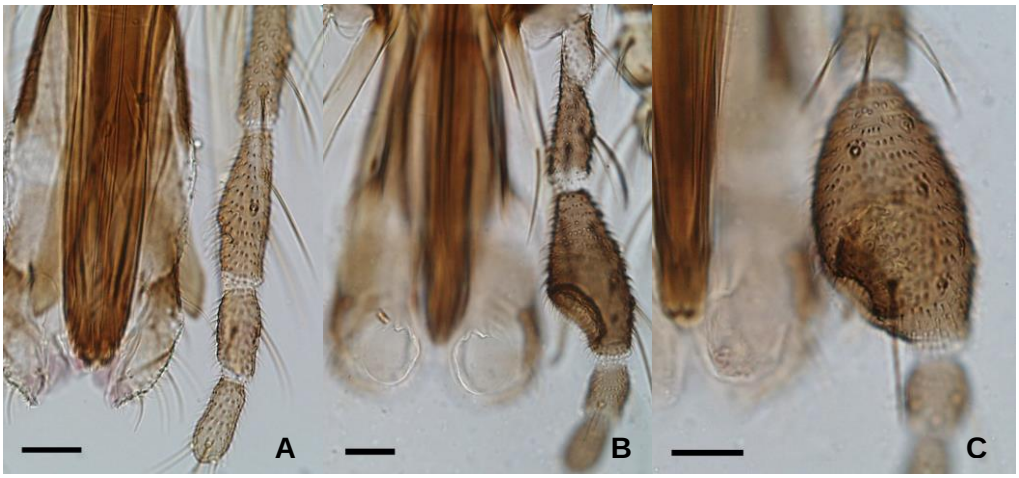
Females have a bigger 3rd palpus segment than males, with different conformations (Figure 1.12.) (Mathieu, 2011):

a) slender or slightly swollen.

b) triangular and moderately swollen.

c) strongly swollen.

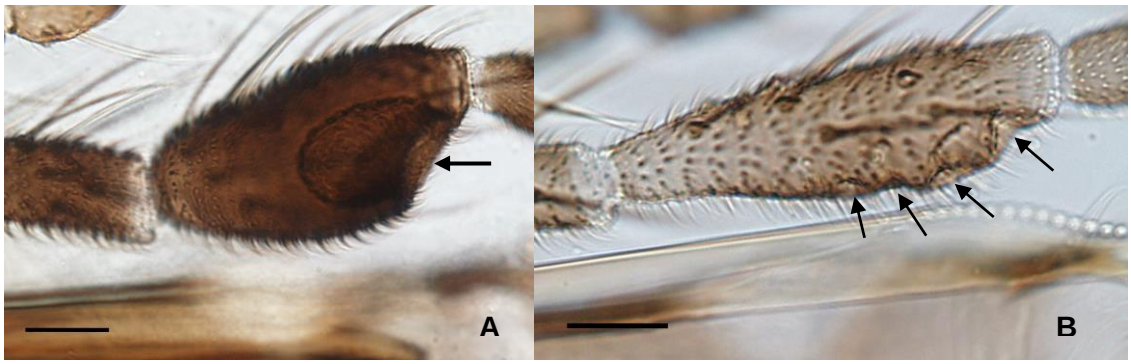
Figure 1.12. – Different conformations of the 3rd palpus segment.



A – *Culicoides impunctatus*: slender; B – *Culicoides gejgelensis*: triangular and moderately swollen; C – *Culicoides begueti*: strongly swollen. Scale bars: 20 µm. Original photos.

Sensorial pit can have different forms, depths or sizes and is an extremely useful morphologic characteristic to distinguish species within *Culicoides* genus (Delécolle, 1985; de Heredia & Lafuente, 2011; Mathieu, 2011): they can be dispersed by the entire 3rd palpus segment (multiple sensorial pits) or localized in its anteromedial region (single sensorial pit) (Mathieu *et al*., 2012; Swanson, 2012; Isberg *et al*., 2013) (Figure 1.13.).

Figure 1.13. – Sensorial pits of two *Culicoides* specimens.



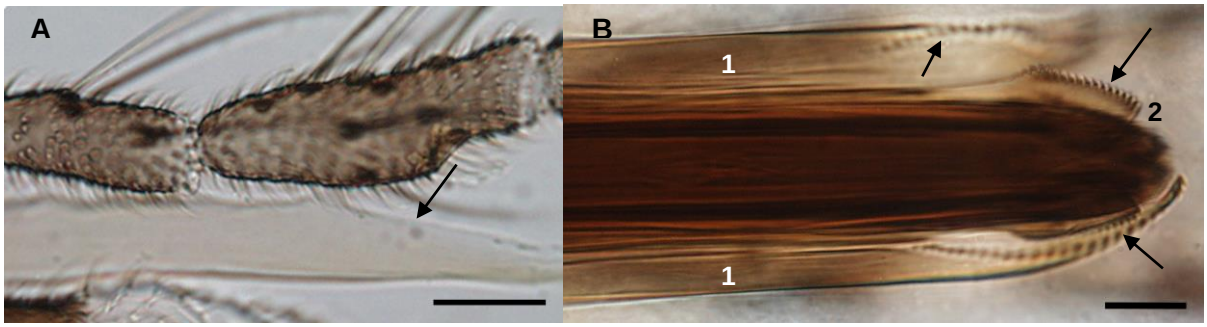
A – Single sensorial pit of *Culicoides circumscriptus* (black arrow); B – Multiple sensorial pits of *Culicoides fagineus* (black arrows). Scale bars: 20 µm. Original photos.

1.5.1.4. Mouth parts

Culicoides proboscis is composed by seven different parts (Delécolle, 1985): one labrum-epipharynx, two mandibles, two maxillae, one hypopharynx and one labium, which wraps all the other mouth parts. Teeth can be present or absent in mandibles and maxillae, according to species (Figure 1.14.) (Mathieu *et al*., 2012).

Female proboscis is more developed and longer than that of males, since the first ones are hematophagous and the second ones phytophagous. Males do not have teeth in both mandibles and maxillae (de Heredia & Lafuente, 2011; Mathieu, 2011).

Figure 1.14. – Mandibles and maxillae of two *Culicoides* specimens.

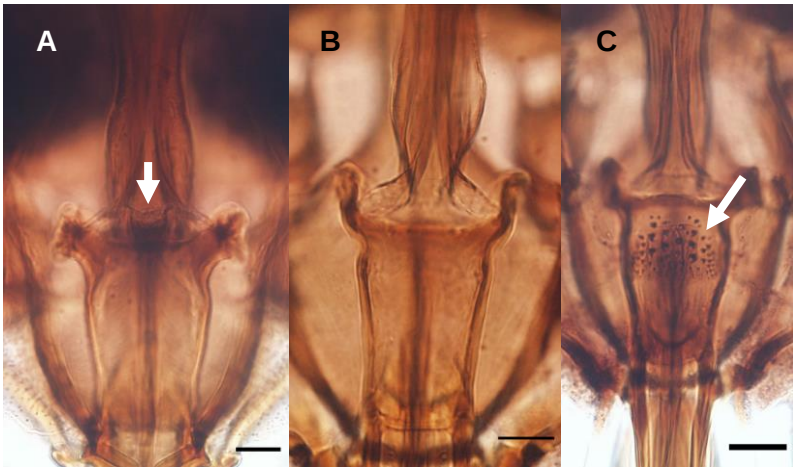


A – Maxille of *Culicoides albihalteratus* without teeth (black arrow); B – Maxillae (1) and mandible (2) of *Culicoides circumscriptus* with teeth (black arrows). Scale bars: 20 µm. Original photos.

1.5.1.5. Pharynx (cibarium) and posterior pharynx

The pharynx or cibarium is a quadrangular structure localized inside the head which unites all mouth parts. In some species, cibarium and/or posterior pharynx can be ornamented with small spicules, feature that can be used to identify some species inside *Culicoides* genus (Figure 1.15.) (de Heredia & Lafuente, 2011; Mathieu *et al*., 2012).

Figure 1.15. – Cibarium and posterior pharynx of three *Culicoides* specimens.



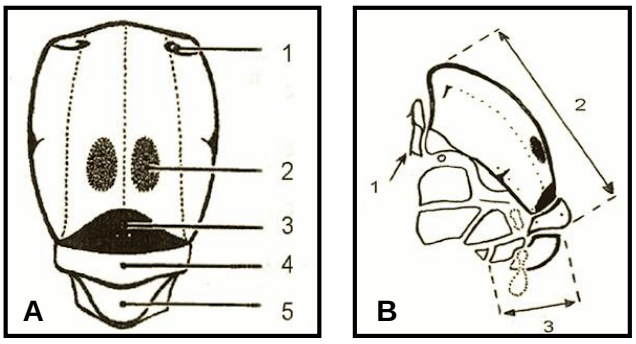
A – Ornamented posterior pharynx (white arrow) of *Culicoides circumscriptus*;
B – Cibarium and posterior pharynx of *Culicoides dendriticus* without ornamentation;
C – Ornamented cibarium (white arrow) of *Culicoides heliophilus*.
Scale bars: 20 µm. Original photos.

1.5.2. Thorax

Adult *Culicoides* thorax (Figure 1.16.) is composed by three segments: a very small prothorax, a mesothorax, which subdivides in three parts: *pre-scutum*, *scutum* and *scutellum*, and a posterior portion, the metathorax (de Heredia & Lafuente, 2011). Furthermore, the thorax comprises a pair of wings, a pair of halters and three pairs of legs. Both *scutum* (the biggest segment of the thorax) and *scutellum* coloration can be useful to distinguish species (Mathieu, 2011). However, this characteristic is very difficult to observe, since it is very

difficult to prepare the thorax horizontally on microscopic slides and their colour and prominences are only visible when the specimen is dry (de Heredia & Lafuente, 2011).

Figure 1.16. – Adult *Culicoides* thorax (adapted from Delécolle, 1985).



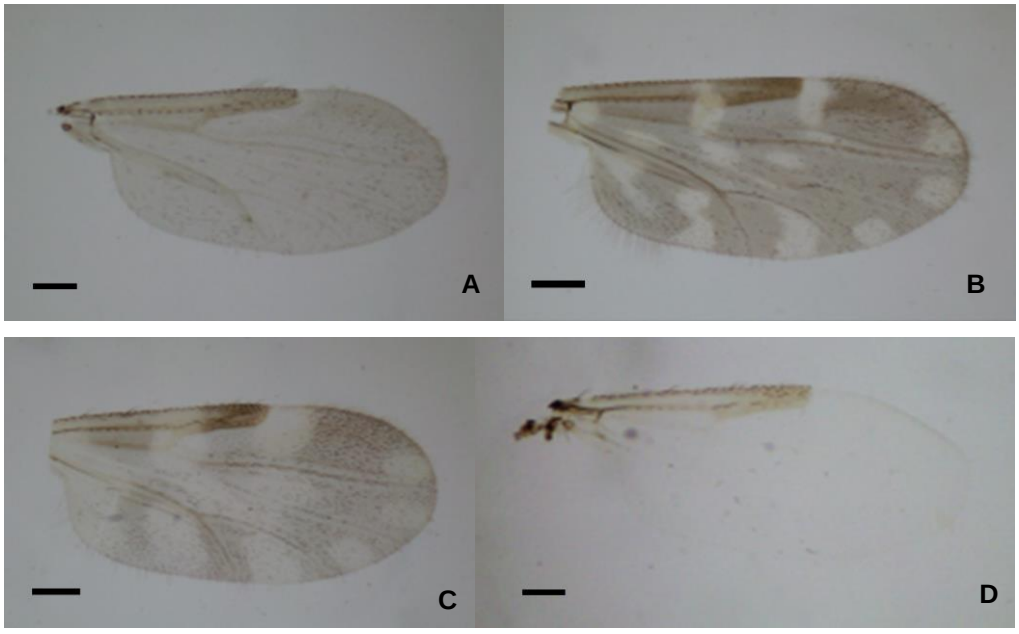
A: Dorsal view. 1 – Humeral impressions; 2 – Scutum patches; 3 – Pre-scutellum pit; 4 – Scutellum; 5 – Metathorax. B: Lateral view. 1 – Prothorax; 2 – Mesothorax; 3 - Metathorax

1.5.2.1. Wings

Wing pattern is the major characteristic used to distinguish *Culicoides* species. *Culicoides* wing is composed of different cells and veins, which are identified by lower and upper case respectively (Figure 1.3., page 7). In males, wings are longer and narrower than female wings (Mathieu, 2011).

All *Culicoides* wings possess two radial cells, characteristic of this genus (Figure 1.4., page 7). Furthermore, some species have dark and light spots or patches, which localization is variable. However, same species have complete dark wings, while others have complete light wings (Figure 1.17.) (Mathieu *et al*., 2012).

Figure 1.17. – Different wing patterns observed in *Culicoides* biting midges.

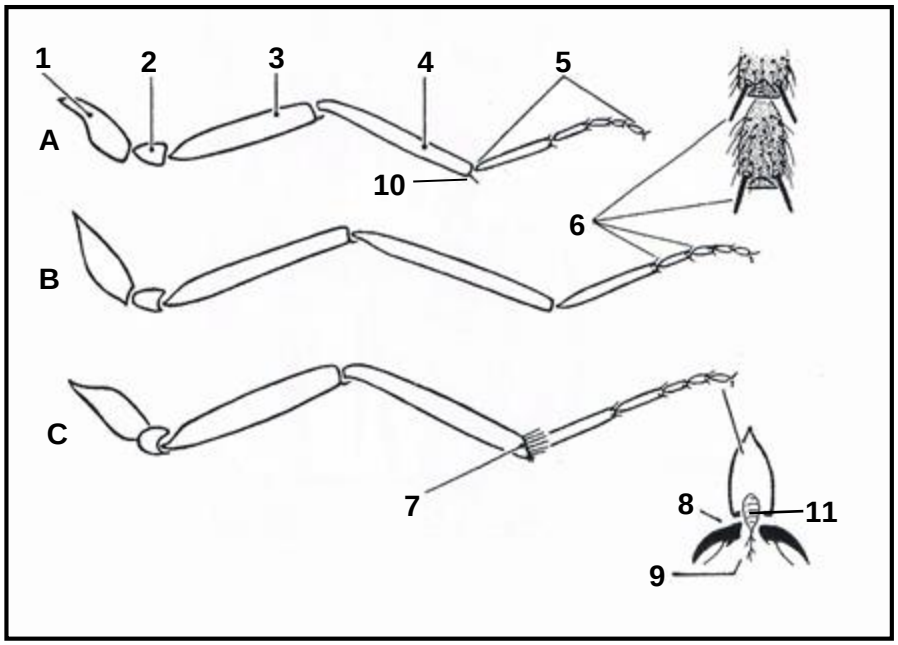


A – *Culicoides corsicus*; B – *Culicoides festivipennis*; C – *Culicoides gejgelensis*; D – *Culicoides heliophilus*. Scale bars: 200 µm. Original photos.

1.5.2.2. Legs

Culicoides biting midges have three pair of legs: fore, middle and hind legs (Figure 1.18.). Each leg is composed by a hip (which connects the leg to the thorax wall), a trochanter, a femur, a tibia, a tarsus (with five tarsomeres numbered from one to five from the proximal to the distal region), a rudimentary empodium on the 5th tarsomere, a little cushion beneath the 5th tarsomere named pulvillus and two claws (Delécolle, 1985; Alexandre-Pires *et al*., 2010; Mathieu *et al*., 2012).

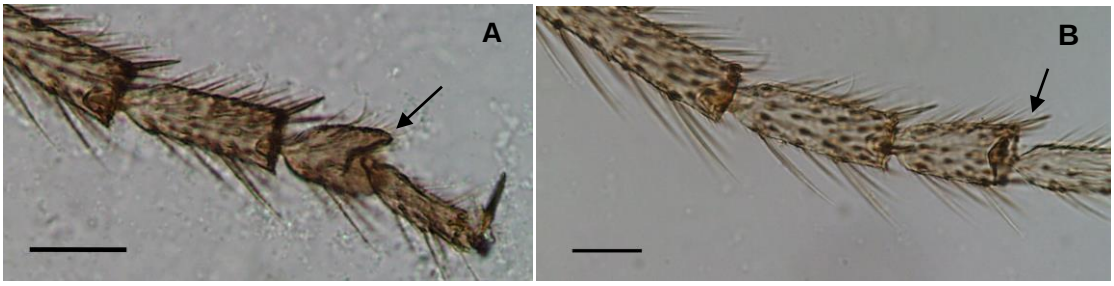
Figure 1.18. – Fore, middle and hind legs of *Culicoides* biting midges (adapted from Delécolle, 1985).



A – Fore leg; B – Middle leg; C – Hind leg; 1 – Hip; 2 – Trochanter; 3 – Femur; 4 – Tibia; 5 – Tarsus; 6 – Tarsal spines; 7 – Tibial comb; 8 – Claw; 9 – Rudimentary empodium; 10 – Tibial spur; 11 – Pulvillus.

All *Culicoides* species possess two small spines from the 1st to the 3rd tarsomere of the middle legs; however, some species also possess two spines in the 4th tarsomere of middle legs, being an useful anatomic characteristic to distinguish some *Culicoides* species (Figure 1.19.) (Mathieu *et al*., 2012). Forelegs have a spur in the distal part of the tibiae and hind legs have a tibial comb in the distal part of tibiae (Mathieu, 2011).

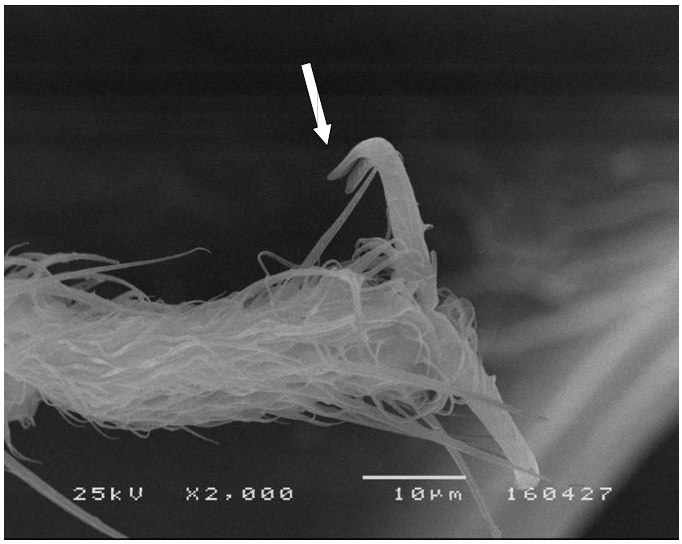
Figure 1.19. – 3rd and 4th tarsomere of middle legs of two *Culicoides* specimens.



A – *Culicoides parroti* without spines on the 4th tarsomere (black arrow); B – *Culicoides pseudopallidus* with spine on the 4th tarsomere (black arrow). Scale bars: 20 µm. Original photos.

Males of Obsoletus group species possess different degrees of bifurcation in their claws and this characteristic can be used to distinguish males inside this group when using scanning electron microscopy (SEM) (Alexandre-Pires *et al*., 2010) (Figure 1.20.).

Figure 1.20. – Bifurcated claw of a *C. obsoletus* male hind leg (Alexandre-Pires *et al*., 2010).



Bifurcated claw marked by a white arrow.

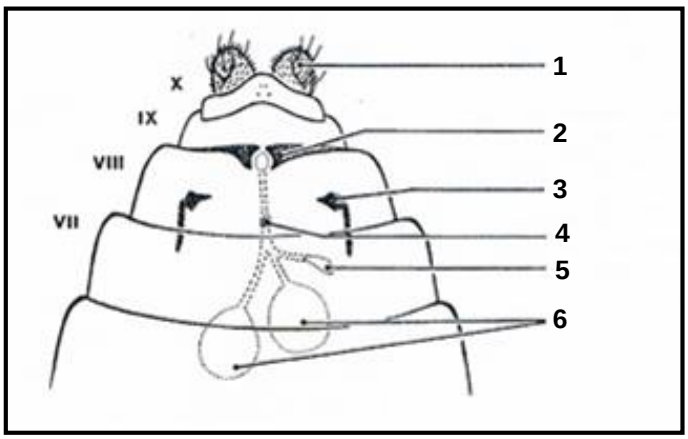
1.5.3. Abdomen

Abdomen of *Culicoides* biting midges is composed by 10 segments (numbered from I to X from the anterior to the posterior region). The distal part of *Culicoides* abdomen has different conformations between females and males (Delécolle, 1985; Mathieu, 2011).

1.5.3.1. Females

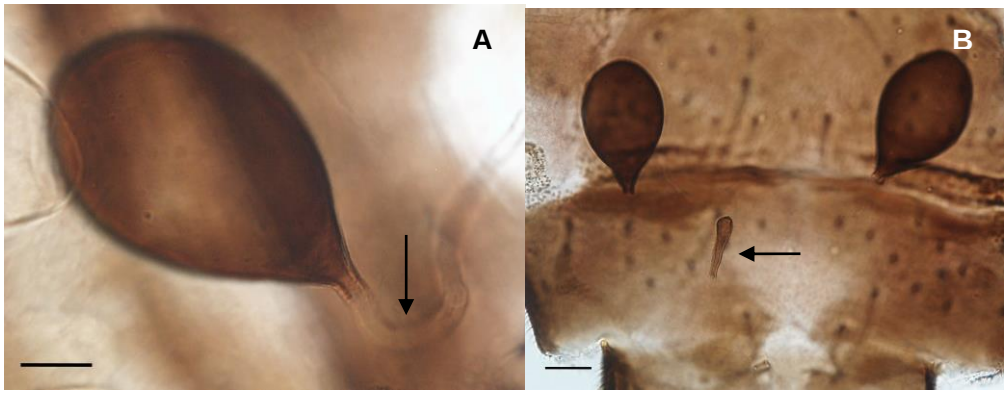
Female *Culicoides* abdomen (Figures 1.21. and 1.22.) have one, two or three functional chitinous spermathecae (where sperm can be stored), depending on species (Delécolle, 1985; de Heredia & Lafuente, 2011; Mathieu, 2011).

Figure 1.21. – Female *Culicoides* abdomen (adapted from Delécolle, 1985).



1 – Cercus; 2 – Chitinous plate; 3 – Abdominal sclerites; 4 – Sclerotic ring;
5 – Rudimentary spermatheca; 6 – Functional spermathecae.

Figure 1.22. – Spermathecae from two *Culicoides* specimens.



A – *Culicoides circumscriptus* single spermathecae, with the spermathecal duct (black arrow).
B – *Culicoides kibunensis*: two functional spermathecae and a rudimentary spermatheca (black arrow). Scale bars: 20 µm. Original photos.

Species that possess two functional spermathecae have always a 3rd non-functional spermatheca, which is smaller than the functional ones (Blanton & Wirth, 1979). Spermathecae shape and number can be used to identify *Culicoides* species (Delécolle 1985). Furthermore, spermathecal duct, which is transparent, can sometimes be visualized (Figure 1.22.).

Some species have a sclerotic ring, which can have different morphological shapes between species (Figure 1.23.). Chitinous plates can sometimes be observed (Delécolle 1985; Mathieu *et al*., 2012). A pair of cerci, which act as sensorial organs, can be visualized in the terminal part of abdomen (Maggenti *et al*., 2005).

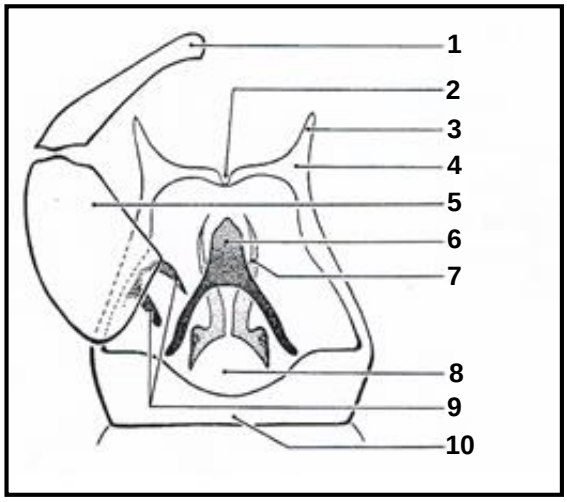
Figure 1.23. – Sclerotic rings from two *Culicoides* specimens.

A – *Culicoides alazanicus*; B – *Culicoides corsicus*. Black arrows: sclerotic rings. Scale bars: 20 µm. Original photos.

1.5.3.2. Males

Male *Culicoides* has a modified abdominal extremity with several structures (Figure 1.24.) which can be used to distinguish species, together with wing pattern (Delécolle 1985).

Figure 1.24. – Male *Culicoides* abdomen (adapted from Delécolle, 1985).



1 – Gonostylus; 2 – Lamellae; 3 – Process; 4 – 9th tergite; 5 – Gonocoxite; 6 – Aedeagus; 7 – Paramera; 8 – Basal membrane; 9 – Dorsal and ventral apodemes; 10 – 9th sternite.

The different structures present in male *Culicoides* abdomen are:

a) The 9th tergite, with a trapezoidal conformation. In some species this structure possesses apicolateral processes in both sides of lamellae.

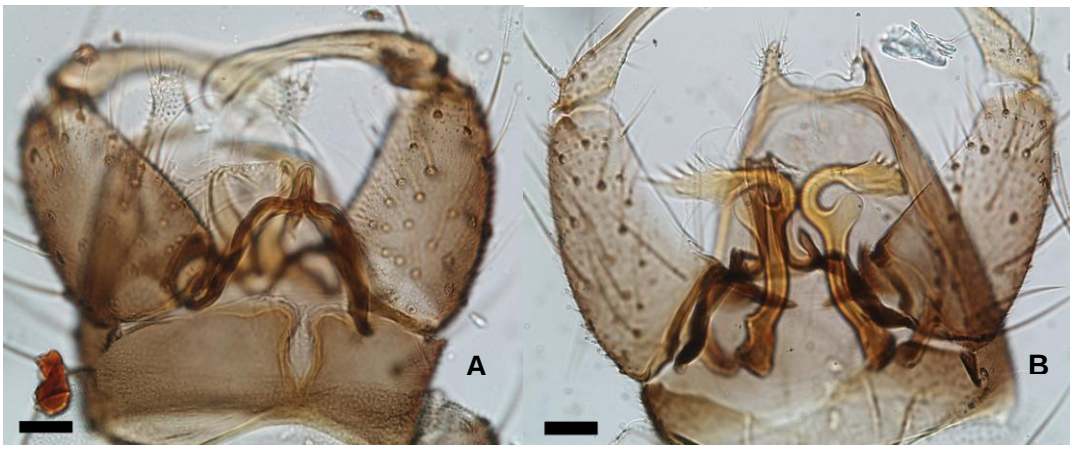
b) The 9th sternite, which is short and can be indented or highly splitted.

c) The basal membrane covering the base of both parameres and aedeagus, which can be with or without small spicules according to species.

d) The aedeagus, an odd structure with a median body and two lateral branches.

e) The parameres, localized under the aedeagus, which components can be united or separated. Aedeagus and parameres can have different conformations and they are very useful to distinguish males of different species (Figure 1.25.).

Figure 1.25. – Abdomen from two males *Culicoides* specimens.



Different conformations of parameres and aedeagus of: A – *Culicoides obsoletus* and B – *Culiciodes semimaculatus*. Scale bars: 20 µm. Original photos.

f) Finally, a pair of styli, inserted basolaterally, which are composed of two parts: gonocoxite and gonostylus. The dorsal and ventral apodemes (invaginations of the cuticle for muscle attachment) are found in the gonocoxite (de Heredia & Lafuente, 2011; Mathieu et al., 2011). Styli have different conformations between species and they are used for grasping the female during copulation (Swanson, 2012).

1.5.4. Morphological alterations in *Culicoides* genus

Although they are not commonly referred in scientific literature, *Culicoides* morphological alterations are of extreme importance since they are related to the quantity or to the aspect of structures with taxonomic importance and this may lead to mistakes during classification. Furthermore, these anomalies can affect insect life activities since they appear in structures with specific functions (Felippe-Bauer & Silva, 2006).

De Heredia & Lafuente (2011) reported three functional spermathecae in 'two functional spermathecae' species (*C. achrayi* and *C. obsoletus*). This particular situation can lead to errors during *Culicoides* species classification, since 'two' and 'three functional spermathecae' species do exist in Nature. Furthermore, the same work detected three specimens with characteristics of both sexes in the same individual (*C. achrayi*, *C. fascipennis* and *C. pictipennis*). These are called intersexual individuals, in which the primary or secondary sexual characters developed to the opposite genre (Smith & Perry, 1967). The most common individuals are those with male genitalia and female antenna, mouth parts and wings, although modified.

Wigglesworth (1950) has shown that anomalous temperatures or internal parasites, both as independent factors, are responsible for changes in insects, causing the formation of intersexual individuals. Sarto i Monteys & Saiz-Ardanaz (2003) found an intersexual *C. circumscriptus* specimen which was parasitized with a mermithid nematode from *Heleidomermis* genus. It appears that parasitized males show more female characteristics than the opposite (de Heredia & Lafuente, 2011).

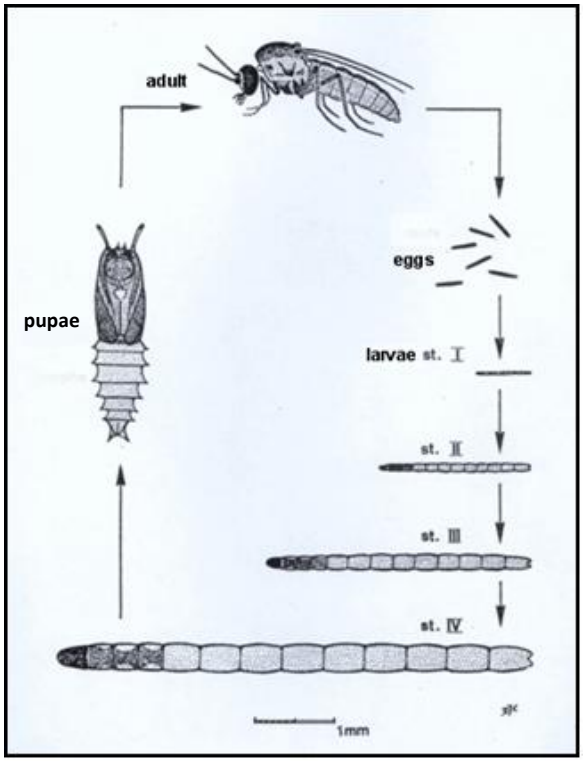
Felippe-Bauer & Silva (2006) also reported different kinds of morphological alterations in several body parts of *Culicoides* species from Central and South America, which they related to genetic or morphogenetic malformation, since no parasitism or sexual anomalies were found. These morphological aberrations included: absence of interocular hairs, fused flagellomeres, double sensorial pits in the 3rd palpus segment, fused palpus segments, deformed mandibular teeth, various wing anomalies, two basal spines in 1st tarsomere, atrophied extra tarsus and three functional spermathecae in 'two functional spermathecae' species.

1.6. *Culicoides* biology

1.6.1. Life cycle

Culicoides biting midges comprise four phases in their life cycle: egg, larvae (with four stages), pupae and adult, being holometabolic insects (Figure 1.26.) (de Heredia & Lafuente, 2011).

Figure 1.26. – Life cycle of *Culicoides* biting midges (adapted from Delécolle).



Culicoides species breed and make oviposition in places where humidity and organic matter are available for larvae development (Zimmer, Losson & Haubruge, 2008). There are many microhabitats for oviposition and posterior development of larvae; as example, lake edges, mud near ponds, forest leaf litter soils, roots and vegetal masses, cattle manure, water contaminated with faeces, sewage systems, swamps, rainwater puddles, rice fields, tree holes, streams and river edges, can be mentioned (Uslu & Dik, 2010).

Culicoides eggs are elongated, with 400-500 µm, and their colour changes from white to black when in contact with air. Oviposition occurs in solid substrate. The number of eggs that each *Culicoides* female can deposit varies from 10 to 675 according to species and environmental conditions (de Heredia & Lafuente, 2011). Eggs are laid in straight line or in small groups where moisture and food are available for larvae development. As examples, *C. circumscriptus* can lay 250 eggs on average (Becker, 1960) while *C. pulicaris* can lay 93 eggs on average (Parker, 1950). Egg hatching occurs between 2 to 8 hours after oviposition. However, it can last exceptionally days or months due to environmental conditions (de Heredia & Lafuente, 2011).

The larva, with a vermiform aspect and bright colour, emerges from the egg. The first instar larvae measure 0.5 mm in length and, after three ecdysis, it reaches the 4th stage, being 10 mm sized in larger species. Larvae have a well sclerotized cephalic region and are legless. They have an apneustic respiratory system.

Culicoides larvae have their optimal development in semiaquatic environments, especially those represented by wet and hot substrates, with high quantities of organic matter (silage residues, dung, wet meadows, muddy roads, river beds, among others) (Goetghebuer, 1952; Zimmer, 2007; Zimmer *et al*., 2008). Frequently, each larval habitat is composed by several *Culicoides* species (Zimmer, 2007) and, usually, *Culicoides* larvae are good swimmers, especially in their later larval stages. They are typically well recognized due to their body undulating serpentine movements in water (de Heredia & Lafuente, 2011). Larvae are localized in the superficial part of the medium where they live, in a depth ranging between 0 and 12 cm, although most of *Culicoides* species are present between 0 and 5 cm, being rare in depths deeper than 8 cm (Uslu & Dik, 2006).

Larval stages duration is variable and depends fundamentally of temperature, and, thus, the period can range between several days to few months. When the 4th larval stage acquires its final size, it develops pupal sketches in thorax and a big quantity of abdominal body fat, being these features those which characterize a 4th stage larva ready to develop to pupa (de Hereida & Lafuente, 2011).

Pupae are usually found in the surface of the environment (mud or water) where the larval development has occurred (Zimmer, 2007). Pupal stage is of short duration (several hours). They can present different behaviour patterns. The most common one is that where pupae rise towards substrate surface and stays there for some time, resting and breathing through

thoracic trumpets, and sinks when any external stimulus occur (de Hereida & Lafuente, 2011).

Finally, adults emerge from pupae, being initially bright and posteriorly acquiring their final colour when their body structures sclerotize. Adults are frequently found in the immediate vicinity of livestock, primarily near wet substrates or standing water. Usually, they don't sparse very much beyond the place of hatching (Mellor *et al*., 2000). Factors such as presence of animals, the proximity of water courses, among others, influence their abundance.

Males emerge faster than females and their sperm is ready in the first 24 hours after hatching. Mating can occur in different ways according to species:

(1) A swarm or a cloud of biting midges appear near aquatic environments or near potential breeding sites. Then, a male specifically recognizes the female by their wing movement, together with sex pheromones. If the female is receptive, mating occurs near the surrounding vegetation.

(2) Without the formation of swarms, male and female localize each other by odour and visual stimuli for posterior mating.

(3) In a less common pattern, male and female are attracted by the same host and male searches for a recent blood engorged female, with which mates and transfers its sperm (de Hereida & Lafuente, 2011). This type of copulation occurs in *C. puncticollis*.

During mating, *Culicoides* male and female are directed in opposite ways, with their abdominal extremity united. For this, male genitalia turns 180° to contact with female genitalia. After insemination, both sexes (but especially females) use their hind legs and their tibial combs to separate from each other (Blanton & Wirth, 1979). In some species, females can accumulate sperm during mating and fertilize several eggs in different oviposition periods. However, other species need to mate several times (Mullen & Durden, 2009). The sperm transfer from a male to a female occurs, in several cases, by intervention of spermatophores, like in *C. nubeculosus*.

Life cycle duration is variable between different *Culicoides* species and is dependent of laboratorial or natural conditions, ranging from one week to more than 3 months.

1.6.2. Circadian rhythm and seasonality

Insect behaviour is directly affected by geophysical (sun and moon light cycles) and climatic (temperature, humidity, wind) factors (Viennet *et al*., 2012). The activity parameters of these biting midges depend on *Culicoides* species, host abundance, season, stabling conditions and weather (Viennet *et al*., 2012).

Usually, *Culicoides* biting midges have crepuscular or nocturnal activity, being at rest during the day, on the lower faces of leaves or in vegetation in shaded areas (Zimmer, 2007; de Heredia & Lafuente, 2011); however, many species also display two biting peaks during the

day: one after sunrise and other close to sunset (*e.g.*, *C. impunctatus*) (Blackwell, 1997; Viennet *et al*., 2012). They start to fly at dusk, taking advantage of temperature drop and humidity rise. In cloudy days or high environmental humidity they can also fly during the day. However, as evening temperatures begin to cool seasonally, adult activity may shift into daylight hours, when temperatures are still warm enough for flight. For exemple, the host-seeking activity of *C. obsoletus* in northeastern Spain during September was greatest in the period immediately before sunset, but peak activity shifted to as much as 90 min before sunset on colder days (Gerry, Sarto I Monteys, Moreno-Vidal, Francino & Mullens, 2009). Diurnal host-seeking activity of crepuscular species has been noted previously (Barnard & Jones, 1980; Mullens, 1995; Carpenter, Mellor & Torr, 2008c) and may be common during cooler weather when nighttime temperatures restrict adult activity. This shift to earlier host-seeking during cool weather may reduce effectiveness of cultural or chemical control techniques applied to reduce biting near sunset, unless timing control measures is similarly adjusted (Mullens *et al*., 2015).

If adult *Culicoides* present at the end of a seasonal pathogen transmission period are surviving in wintertime resting sites, waiting for a suitable flight temperature, the first warm day could pose considerable risk of pathogen transmission for animals in the vicinity of these resting sites (Mayo *et al*., 2014; Mullens *et al*., 2015).

While there are some species which prefer specific night hours, other species have diurnal habits, like *C. dewulfi* and *C. heliophilus* (Kettle, 1962a). Midges from Obsoletus group have a main activity peak around sunset and can also have a main activity peak at sunrise (van der Rijt, van den Boom, Jongema & Oldruitenborgh-Oosterbaan, 2008; Sanders *et al*., 2012; Viennet *et al*., 2012).

The density of adult *Culicoides* populations varies according to seasons. In fact, some species have a wider distribution during the year while others are found only during a short time (Zimmer *et al*., 2008). As example, *C. impunctatus* appears from May to September (Service, 1971) while *C. obsoletus* and *C. scoticus* appear earlier and have a longer flight period; they appear continuously from early April to mid-December (Rieb, 1982; Meiswinkel *et al*., 2014b). In general, two generations per year can be observed, one in spring and other in summer (Rieb, 1982). The seasonality of disease outbreaks is associated with the timing of the annual peak in vector numbers (Howell, 1979; Herniman, Boorman & Taylor, 1983; Elfatih, Mohammed & Taylor, 1987; Mohammed & Mellor, 1990; Baylis, El Hasnaoui, Bouayoune, Touti & Mellor, 1997).

Seasonal periods of peak abundance overlap in most *Culicoides* species and there is also evidence of temporal segregation over the 24h cycle (Viennet *et al*., 2012). Several species can co-occur, although not co-dominantly, at the same sampling site (Blackwell, Mordue (Luntz), Young & Mordue, 1992b). Scolamacchia, van den Broek, Meiswinkel, Heesterbeek & Elbers (2014) referred the occurrence of up to 22 *Culicoides* species in these conditions,

number that is considerably lower than those observed in tropics, possibly reflecting a lower niche diversity at more temperate latitudes. *Culicoides* seasonal demography and breeding habitats probably have importance in the predominance of certain species over others in specific areas. Furthermore, microhabitats, artificial breeding sites near traps and farm management can probably have influence in *Culicoides* life cycle (Scolomacchia *et al*., 2013).

Black-light suction traps are the standard method for collecting *Culicoides* worldwide and they are considered to represent proportionally the size of the local midge populations found at the sampling site, multiplied by the activity rate and trap efficiency (Carpenter *et al*., 2008a; Viennet *et al*., 2011; Scolamacchia *et al*., 2014), as well as *Culicoides* biodiversity, mapping their seasonal and geographic ranges.

Finally, *Culicoides* vectors activities usually reduces or ceases with low temperatures and BTV transmission, in many temperate regions, is almost completely interrupted for several months of the year by cold weather. However, outbreaks often resume after these interruptions far longer than the typical lifespan of an adult vector or the normal period of host infectiousness. This phenomenon is defined as overwintering (Wilson & Mellor, 2009) and, although several mechanisms have been proposed for it (Nevill, 1971), how viral interseasonal maintenance occurs is yet to be found.

Takamatsu, Mellor, Mertens, Kirkham, Burroughs & Parkhouse (2003) demonstrated that BTV can persistently infect ovine γδ T-cells *in vitro*. This process may also occur during infection and viremia in mammalian hosts, thus providing a mechanism for virus persistence. Chatzopoulos *et al*. (2015) suggested that the virus survival process may involve an additional host, capable of viral reintroduction into the insect population. The distribution and expansion of the BTV in northern European countries is a typical example of overwintering, which might have occurred with contribution of wildlife.

1.6.3. Larval feeding behaviour

Larval feeding behaviour presents a high variety of patterns according to the environment where they develop. In a general way, two types of feeding pattern can be distinguished and this factor will affect the morphology and physiology of their dental structures. Some species feed of nematodes, oligochaetes, rotifers, immature stages of other insects, etc., while others feed of debris, organic materials and microorganisms (bacteria, fungi, protozoa, algae and diatoms) (de Heredia & Lafuente, 2011). However, based in laboratorial experiments and direct observations, most *Culicoides* species are omnivores in their larval stages and they feed on any type of available organic matter (de Heredia & Lafuente, 2011).

1.6.4. Adult feeding behaviour

Females from *Culicoides* genus are essentially hematophagous and they feed on several vertebrate hosts, like mammals, birds, reptiles and amphibians (Ronderos, Díaz & David, 2004; Votýpka, Synek & Svobodova, 2009), while males are exclusively phytophagous, feeding on flower nectars (de Heredia & Lafuente, 2011). When feeding, females rupture the skin with their mandibles to reach blood vessels and blood flows to the adjacent tissues; then, the insect, aimed by a pharyngeal pump, sucks the blood into its digestive system, in a process called telmophagy. Once finished the blood meal, the female rests in surrounding vegetation for several days during eggs development (Mullen & Durden, 2009).

Most female *Culicoides* need blood to reach sexual maturation and to make oviposition (anautogenous species). However, some species, like *C. impunctatus* or *C. circumscriptus*, are autogenous and do not require a blood meal to make their first oviposition (Boorman & Goddard, 1970; Blackwell *et al*., 1992b); besides, there are also some parthenogenic species (de Heredia & Lafuente, 2011). However, females can also feed on flower or plant sugars and also from aphids to obtain alimentary supplements for their flight activity (Chaker, 1983).

Concerning their feeding behaviour, *Culicoides* species can be classified as endophagous or exophagous, according to the local where the blood meal was taken, respectively inside or outside human-made structures (Clements, 1999). This factor is extremely important concerning control methods and animal protection. Usually, the number of species captured inside stables is lower than those captured outside (Baldet *et al*., 2008; de Heredia & Lafuente, personal observation, 2009). However, the degree of exophagous behaviour is temperature dependent and, in this way, more specimens are captured outside than inside buildings in summer, while in the Fall, due to the wind and less favourable temperatures, *Culicoides* tend to be captured inside stables. On the other hand, Viennet *et al*. (2012) observed that *C. obsoletus* or *C. scoticus* have some degree of endophagy, although Palearctic *Culicoides* species are primary exophagous insects. In South Africa, *C. imicola* has a nocturnal and mainly exophagic behaviour (Barnard, 1997; Meiswinkel *et al*., 2000).

Aspiration of *Culicoides* biting midges is a preferred method instead of black-light traps for establishing accurately how many midges feed on an animal host (Dzhafarov, 1964a; Scheffer *et al*., 2012), since their behaviour regarding attraction is different (Kirkeby, Græsbøll, Stockmarr, Christiansen & Bødker, 2013).

According to some authors, *Culicoides* biting midges do not have a specific host and they follow an opportunistic behaviour, feeding on the more accessible or on the closest one (de Heredia & Lafuente, 2011). Molecular studies that have been performed to investigate the feeding pattern of *Culicoides* have shown that (Annex 1.3.) (Garros *et al*., 2011; Martínez-de la Puente, Figuerola & Soriguer, 2015):

(1) Most of the studied species are able to feed on several vertebrate species.

(2) Although some species feed primarily on either mammals or birds, this is not a strict behaviour, since some species also feed on animals of their non-preferred vertebrate group.
(3) Taxonomically or phylogenetically related species tend to feed on the same classes of vertebrate hosts.

1.6.4.1. Host preferences

Several species feed preferentially in domestic and wild mammals, like *C. imicola*, species belonging to Obsoletus and Pulicaris groups and *C. puncticollis* (Blackwell, Mordue & Mordue, 1994; Foxi & Delrio, 2010; Linden *et al*., 2010). Both Obsoletus and Pulicaris groups are strongly associated with livestock farm habitats (Takken *et al*., 2008) and they can also feed occasionally in birds (Martínez-de la Puente *et al.,* 2015). There are also ornithophilic *Culicoides* species, whose breeding sites coincide with bird habitats. *C. circumscriptus*, *C. cataneii, C. festivipennis*, *C. simulator, C. sahariensis* and *C. univittatus* are examples of exophagous ornithophilic species (Blackwell *et al*., 1994; Martínez-de la Puente *et al,* 2009; Foxi & Delrio, 2010).

Fall *et al*. (2015) have shown that species belonging to *Avaritia* subgenus have horses as preferred host when compared to sheep. However, *C. obsoletus* and *C. scoticus* are attracted by and have their blood meal on cattle, sheep, goat, horse, swine and lagomorphs (Carpenter *et al*., 2008a; Gerry *et al*., 2009; Mullens, Gerry, Monteys, Pinna & González, 2010; de Heredia & Lafuente, 2011; Ninio, Augot, Delécolle, Dufour & Depaquit, 2011a). Ninio *et al*. (2011a) have also found wild boar blood in a *C. scoticus* engorged female. *C. dewulfi* can also feed in cattle, sheep and wild boars (Ninio *et al*., 2011a, Martínez-de la Puente *et al*., 2015), *C. chiopterus* in cattle and horses (Dijkstra, van der Ven, Meiswinkel, Holzel & Van Rijn, 2008; Ninio *et al.,* 2011a) and *C. imicola* on horses, cattle and sheep (Logan, Cook, Mordue (Luntz) & Kline, 2010).

Bartsch, Bauer, Wiemann, Clausen & Steuber (2009) found that species belonging to Pulicaris group feed on red deer. *C. pulicaris* shows preference for lagomorphs and *C. lupicaris* feeds in cattle, pigs and, less frequently, in horses and lagomorphs (Ninio *et al*., 2011a). Ninio *et al*. (2011a) also found *C. lupicaris* specimens that had fed in wild boars.

Multiple hosts have an important role on abundances and prevalence of vector species. Calvo *et al*. (2012) have shown that, on average, each *Culicoides* species can feed on the blood of 5 different host species. The most extensively sampled *Culicoides* species feed in both bird and mammal vertebrates. Together with *C. kibunensis* and *C. festivipennis*, this group includes *C. imicola* and members from Obsoletus and Pulicaris groups.

C. picturatus, *C. achrayi* and *C. pallidicornis* can feed in cattle and lagomorphs, being these two last species capable of doing their blood meals on cattle inside stables (Ninio *et al*., 2011a). *C. parroti* feed on horses and sheep (Mellor & McCaig, 1974; Gerry *et al*., 2009) and

C. impunctatus feed predominantly in sheep, cattle and deer, although they also frequently feed in humans (Mands, Kline & Blackwell, 2004).

1.6.5. Vector competence and vector capacity

Vector competence and vector capacity are two important concepts to take into account concerning vector-borne diseases (Gerbier *et al*., 2007). The first one is defined as the innate ability of a vector to acquire a pathogen, maintain it and successfully transmit it to a susceptible host (Macdonald, 1957; Garret-Jones, 1964; Purse *et al*., 2005). Concerning viruses, vector competence may be proved in laboratorial experiments by providing groups of insects of a specified species with blood meals of appropriate concentrations of virus, assessing the infection and transmission rates. Thus, another definition of vector competence is the proportion of feeding insects that support virus replication and transmit it after a suitable incubation period (Saegerman *et al*., 2008). Due to refeeding difficulties concerning *Culicoides* species, it has been assumed that viral transmission occurs if virus can be recovered from salivary glands.

Vector capacity is defined as the potential for virus transmission of an insect population (disease transmission risk), taking into account a range of insect, host and environmental variables, including vector abundance, vector survival, biting and transmission rates, host preferences and host abundances, under a range of external conditions (*e.g.*, bioclimatic) (Saegerman *et al*., 2008).

Probably not all *Culicoides* species are competent vectors of BTV and the reason beyond this situation is complex (Mellor, 2000). Shortly, when an arbovirus like BTV is ingested it passes into the lumen of the mid-gut hind part. Then it has to gain access to the body of the insect properly before the potentially hostile environment in the gut lumen inactivates it or before it is excreted. Since BTV is transmitted orally by the vector, it must reach the salivary glands with or without amplification in other susceptible tissue, multiply in them and finally be released with saliva into the salivary ducts where it is available to infect a second vertebrate host during a subsequent bite (Mellor, 2004). The details of this cycle (duration, infected tissues, viral titre produced, proportion of infected insects and transmission rate) are controlled by a range of interdependent variables, such as the virus, the insect host and environmental factors (especially temperature). The main constraints or barriers for BTV to infect *Culicoides* or to be transmitted to vertebrate hosts by non-vector *Culicoides* species and even within a variable proportion of individuals within vector species are:

(1) Infection of the mid-gut cells (mid-gut infection barrier).

(2) Escape of viral progeny from the mid-gut cells into the haemocoel (mid-gut escape barrier).

(3) Viral dissemination through haemocoel to the salivary glands (and ovaries if transovarial transmission occurs) (dissemination barrier), which may vary from a few days to several

weeks or more, depending on temperature and viral serotype (Mullens, Tabachnick, Holbrook & Thompson, 1995; Wittmann, Mellor & Baylis, 2002).

(4) Infection of the salivary glands (salivary gland infection barrier).

(5) Release from the salivary glands into the salivary ducts (salivary gland escape barrier) (Mellor, 2004).

For *Culicoides* vector species, when midges are placed in high temperatures, infection rates are higher, rates of virus production are faster and transmission occurs earlier than with low temperatures. However, midges survive for a shorter time when compared with those submitted to low temperatures (Mullens *et al*., 1995; Wellby, Baylis, Rawlings & Mellor, 1996). BTV requires a minimum temperature of 10 °C to 15 °C to replicate once ingested by a *Culicoides* vector (Wittmann *et al*., 2002; Wilson, Carpenter, Gloster & Mellor, 2007) although it can persist in vectors at cooler temperatures, resuming replication when conditions are favourable (Mullens *et al*., 1995; Paweska, Venter & Mellor, 2002). The time required by an insect vector to digest a blood meal is also reduced at higher temperatures, increasing the frequency of blood-feeding (Wilson & Mellor, 2009).

Some species (like the “non-vector” species *C. nubeculosus*) have an oral susceptible rate of less than 1% when placed at 25 °C. However, if temperature is raised to 30 °C or 35 °C, their oral susceptible rate increases above 10% and viral replication also increases to levels where transmission is possible (Mellor, Rawlings, Baylis & Wellby, 1998; Wittmann, 2000). Boorman (1960) stated that an increase in developmental temperature not only gives rise to smaller adults but to adults with an increased incidence of “leaky gut” phenomenon. This phenomenon was also observed by Tabachnick (1996), where there was a similar increase in infection rates in small adults, brought by poor larval nutrition. In *C. nubeculosus* females with a “leaky gut”, virus may be able to cross directly from the ingested blood meal in the gut lumen into the haemocoel without first infecting and replicating in gut cells (Mellor *et al*., 1998) and, once in haemocoel, most arboviruses replicate and may be transmitted, even by normally non-vector insects. Increasing temperature due to climate change could increase the likelihood of such individuals, inducing competence in otherwise non-competent species (Mellor *et al*., 2000; Wilson & Mellor, 2008).

The mortality rate of adult *Culicoides* is equally important in determining the proportion of vectors that survive, once they ingest the virus, so it can reach the salivary glands (extrinsic incubation period). Studies have shown that adult mortality in *Culicoides* is affected by temperature (Hunt, Tabachnick & McKinnon, 1989; Gerry & Mullens, 2000; Wittmann *et al*., 2002) and probably by humidity (Wittmann *et al*., 2002). Precipitation also affects the activity patterns of adult *Culicoides* and the seasonal availability and stability of breeding sites (Wilson & Mellor, 2008).

Culicoides species belonging to *Avaritia* subgenus are implicated as the primary vectors of BTV and SBV above the Mediterranean region, based on their abundance and host

preference (Garros, *et al*., 2011; Venail *et al.,* 2012; Viennet *et al*., 2013; Meiswinkel *et al*., 2014a), vector competence studies (Carpenter, Lunt, Arav, Venter & Mellor, 2006; Carpenter *et al*., 2008b; Veronesi *et al*., 2013) and isolation or detection of virus in field-collected midges (Mellor & Pitzolis, 1979; Savini *et al.*, 2005; Meiswinkel, van Rijn, Leijs & Goffredo, 2007; Djikstra *et al*., 2008; Hoffman *et al.,* 2009; Elbers *et al.,* 2011; Venail *et al.*, 2012; de Regge *et al*., 2013).

Scolamacchia *et al*. (2014) have shown that *C. pulicaris*, along with its very low abundances in certain areas of Netherlands, is unable to penetrate into all areas and to satisfy the three inter-related elements [abundance, seasonal persistence and several generations per year (multivoltinism)] that a competent vector appears to possess, unlike biting midges of *C. punctatus* and *C. newsteadi* species, which appear all year and gather all the conditions mentioned above. Recently, BTV genome was found in these two last species (Goffredo *et al*., 2015), showing that they can be potential vectors of BTD.

1.6.6. *Culicoides* saliva

The study of bioactive molecules and antigens in *Culicoides* saliva is an increasingly important area of research, both in understanding their impact on arboviruses transmission between vector and host and in examining the immunological response of the host to the biting activity (Rádrová *et al*., 2015).

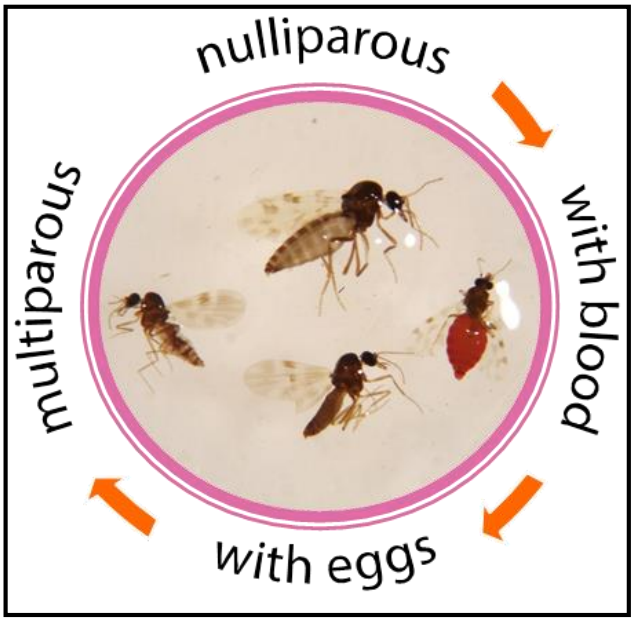
Darpel *et al*. (2011) have shown that the treatment of BTV particles with saliva collected from the BTV vector *C. sonorensis* lead to the formation of highly infectious subviral particles. In addition, it was also demonstrated that the feeding activity of *Culicoides* can increase the titer of BTV-infected host viremia and the severity of clinical signs in sheep (Pages *et al*., 2014).

Furthermore, *Culicoides* saliva has been found to contain powerful allergens, leading to an immunoglobulin E-mediated type 1 hypersensitivity response that occurs in livestock after *Culicoides* bites (Yeruham, Braverman & Orgad, 1993; Wilson, Harwood, Björnsdottir, Marti & Day, 2001).

1.6.7. Female parity status

Female parity or oviposition status varies with abdominal pigmentation in *Culicoides* genus (Dyce, 1969). Parous (or multiparous) females have a darker, burgundy-red pigment and have completed at least one gonotrophic cycle (blood fed and laid eggs), while nulliparous females have a lighter abdominal color and have not yet blood fed or laid eggs (Figure 1.27.). The parity status thus provides a relative estimate of the female's age and reproductive success (Morag, Mullens & Gottlieb, 2013). Parity status, together with seasonal patterns of *Culicoides* abundance and daily survival, is important for assessing the potential for pathogen transmission in an area (Lysyk, 2007).

Figure 1.27. – *Culicoides* female parity status (Marques *et al*., 2013).



1.7. Factors influencing *Culicoides* occurrence

The main factors influencing the occurrence of the different stages of *Culicoides* biting midges are the following: climatic variables, edaphic, topographical and host availability factors (Calvete *et al*., 2009; Silbermayr, Hackländer, Doscher, Koefer & Fuchs, 2011; Purse *et al.,* 2012). However, viral diseases outbreaks and their rapid spread shows the lack of knowledge about these vectors, in particular their distribution, larval ecology and, mainly, host preferences (Zimmer, Verheggen, Haubruge & Francis, 2015). There are other factors less mentioned in literature that also influence *Culicoides* occurrence: manure storage, presence of water sources, presence and type of vegetation surrounding the farm and the pasture (open or wooded), the distance of the light trap from the livestock, light intensity, lunar cycles, relative humidity and pressure changes (de Heredia & Lafuente, 2011; Rigot, Drubbel, Delécolle & Gilbert, 2013; Kluiters *et al*., 2013).

1.7.1. Climatic variables

Climatic variables, together with other environmental factors, are known to modulate *Culicoides* species lifecycle, activity and survival and have been linked to the timing and distribution of BTD outbreaks at different scales (Purse, Brown, Harrup, Mertens & Rogers, 2008; Zimmer, *et al*., 2008).

As bioclimatic data is important to define *Culicoides* geographical distribution and temporal abundance, this information has been adopted to complement data survey and also for the development of climate-based models for prediction of vector distribution in non-surveyed locations and for identification of areas at risk of BTD outbreaks (Baylis, Mellor, Wittmann & Rogers, 2001; Wittmann, Mellor & Baylis, 2001; Tatem *et al*., 2003; Purse *et al*., 2004a; Scolamacchia *et al*., 2014). From all climatic variables, those temperature-related, humidity

and wind were confirmed as the main factors which influence *Culicoides* behaviour, survival, activity and dispersal as measured by suction light traps (Peng, Fletcher & Sutton, 1992; Baylis, Bouayoune, Touti & El Hasnaoui, 1998; Purse *et al*., 2004b; Zimmer *et al*., 2008). However, there is still a lack of basic knowledge concerning *Culicoides* ecology (*e.g.*, breeding or resting sites) and, in this way, the comprehensive interpretation of the modelling results is often subject to uncertainties (Lühken, Steinke, Wittmann & Kiel, 2014).

Accuracy in temperature measurements is extremely important, for exploring the possible effects of global warming on ecosystems and organisms (Logan, Régnière & Powell, 2003; Gutierrez, Ponti & Cossu, 2009; Ponti, Cossu & Gutierrez, 2009) and for developing strategies in order to manage vector-borne diseases of humans and animals over large geographic areas (Rogers, Hay & Packer, 1996; Gilioli & Mariani, 2011; Amek *et al*., 2012; Chen & Hsieh, 2012).

Culicoides activity is significant between 13 °C and 35 °C (Braverman & Chachik, 1996), although these values vary between species (Zimmer *et al*., 2008). Temperature influence and preference according to species is referred in some works. *C. chiopterus* larvae can survive with temperatures below the freezing point. Steinke, Lühken & Kiel (2015) have shown that *C. chiopterus* larvae emergence can occur with temperatures of -18 °C, although the same is not observed for *C. dewulfi*. In this way, the divergent distribution of these two species in countries with extreme temperatures (*e.g.*, Siberia, where only *C. chiopterus* is present) can be explained by the different resistance of these two species to freezing (Sprygin *et al*., 2014; Steinke *et al*., 2015). Although the reason for this resistance for sub-zero temperatures is not known, Vaughan & Turner (1987) reported high levels of the cryoprotectant glycerol (the most common substance in insects which undergo overwintering) (Lee Jr, Lee & Strong-Gunderson, 1993) in winter larvae of other *Culicoides* species when compared with those collected in summer.

Furthermore, Nunamaker (1993) showed that some *Culicoides* species have a rapid physiological adaptation to low temperatures. In countries with very cold winter weathers, *Culicoides* are not found outside buildings but inside, where they can take shelter, allowing eggs and larvae survival (de Heredia & Lafuente, 2011). Moreover, *C. obsoletus* can fly inside barns with temperatures ranging between 6 °C and 12 °C during winter (Losson *et al*., 2007). Many *Culicoides* species overwinter in temperate regions in the larval (Becker, 1960; Jones, 1967; Szadziewski, Krzywinski & Gilka, 1997; White, Wilson, Blair & Beaty, 2005) and adult stages (Rawlings & Mellor, 1994; Gerry & Mullens, 2000; Venter *et al*., 2014). As examples, *C. chiopterus* and *C. dewulfi* larvae can be found overwintering in cowpats on cattle pastures (Kettle & Lawson, 1952; Steinke, Lühken & Kiel, 2014) and overwintering of adults has been demonstrated in mild climates (Gerry & Mullens, 2000).

High humidity is also an important feature concerning *Culicoides* development and survival (Murray, 1991). Larvae are particularly sensible to drying, killing them quickly. Drought is also

unfavourable to adults, who refuge in vegetation instead. When the weather is stormy and rainy they resume their activity. These circumstances justify the reason why these vectors are abundant in late summer/early autumn in temperate regions (Zimmer *et al*., 2008).

Sellers (1992) observed two types of flight in *Culicoides* species: (1) short-distance flights, that can occur in any direction (both up- and downwind) and at low or zero wind speeds and (2) long-distance dispersal, up to several hundred kilometres, that occurs at wind speeds greater than the flight speed of *Culicoides* (windborne dispersal), due to their small size (de Heredia & Lafuente, 2011).

Most adult *Culicoides* species move passively over very short distances, usually a few hundred meters to up to 2 km from their breeding sites by hot and humid winds of low altitude, blowing at medium speed (from 10 to 40 km/h) and, in this way, their dispersion is very limited (Mellor *et al*., 2000; Zimmer *et al*., 2008). However, windborne dispersal can occur over much longer distances according to some authors, like hundreds of kilometres due to prevailing winds (Sellers & Maarouf, 1989, 1991; Sellers, 1992; Braverman & Chechik, 1996), leading to a rapid spread of the diseases that they might carry (Hendrickx *et al*., 2008); outbreaks of BTD in the Mediterranean, North Africa and northern Europe, AHSD in the Middle East and EHD in Israel have been attributed to this means of dispersal (Sellers, Pedgley & Tucker, 1977; Braverman & Chechik, 1996; Ducheyne *et al*., 2007; Gloster, Burgin, Witham, Athanassiadou & Mellor, 2008; Agren, Burgin, Lewerin, Gloster, Elvander, 2010; García-Lastra *et al*., 2012; Eagles, Walker, Zalucki & Durr, 2013). These long-distance movements may not be accidental, since the insect can play a role on it by actively initiate and maintain the movement (Dingle, 1996). These movements may be terminated (when midge lands) either actively (by ceasing wing movement and descending), when wind drops (Sellers, 1992) or due to terrain topography (Bishop, Barchia & Spohr, 2000; Bishop, Spohr & Barchia, 2004). In the case of BTD, once landed, the midge must survive long enough to replicate the virus to a transmissible level and to bite a susceptible ruminant host. This last feature is influenced by the local habitat, weather conditions and presence of hosts at destination (Hendrickx *et al.,* 2008).

1.7.2. Edaphic and topographical factors

1.7.2.1. Larvae

Larval development sites of different *Culicoides* species are particularly varied and generally species-specific (Zimmer *et al*., 2014). Soil moisture is expected to be an important factor for *Culicoides* species occurrence, although the link between it and *Culicoides* larval development needs more experimental attention (Mellor *et al*., 2000), since a deeper understanding would help to interpret different patterns of species distribution (Lüken, Steinke, Wittmann & Kiel, 2014).

Larvae of biting midges belonging to *Avaritia* subgenus usually have a slow head-to-tail flexion, in contrast to other *Culicoides* species movements, which are serpentine-like (Lüken *et al*., 2014). Furthermore, their pupae are not able to float (Cannon & Reye, 1966) and their habitats are described as moist but not waterlogged (Nevill, Venter, Meiswinkel & Nevill, 2007). This can explain the adaptations of these larvae to breeding sites with high viscosity (*e.g.*, dung) and the difference observed when compared with other species (Lüken *et al*., 2014).

1.7.2.2. Pupae

According to the different breeding sites, pupae can be classified into four different categories (Dyce & Murray, 1966): type A pupae are able to float after flooding but are not able to submerge again and breed on the margins of still and slow flowing waters; type B pupae breed on tree holes and can variably float or submerge; type C pupae remain submerged and burrow in the substrate as adaptation for breeding sites in estuarine sands, which are influenced by flooding or desiccation according to tide; type D pupae cannot float or burrow and lie on the substratum, drowning if flooded (Nevill, 1967; Foxi & Delrio, 2010).

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1.7.2.3. Adults

The developing and breeding sites of adult *Culicoides* are still poorly known (Zimmer, Losson, Saegerman, Haubruge & Francis, 2013a). They can breed in a wide range of soils as long as they provide enough moisture and organic matter for larvae development (Kettle, 1962b). According to Meiswinkel, Venter & Nevill (2004b), the diversity of *Culicoides* breeding sites can be grouped into three major categories: water saturated soil between aquatic and terrestrial habitat, fresh dung pats and moist, decaying organic matter (including manure). *Culicoides* species prefer soils which absorb water very slowly and retain moisture and nutrients and avoid those related to arid or better drained areas (Scolamacchia *et al*., 2014).

1.7.2.4. Species habitat preferences

Larvae of species from Obsoletus group prefer wet forest litter (Glushchenko & Mirzaeva, 2008) as well as stagnant water reservoirs and marshes with dense vegetation (Dzhafarov, 1976).

C. obsoletus and *C. scoticus* are negatively linked to arable areas and prefer forest leaf litter soils (Conte, Goffredo, Ippoliti & Meiswinkel, 2007); however, some have shown that these two species can breed in dried dung left in cowshed walls (Zimmer, Saegerman, Losson & Haubruge, 2010; Ninio, Augot, Dufour & Depaquit, 2011b). It is also known that these two species can breed in maize silages usually located close to livestock buildings (Zimmer *et al*., 2008).

C. chiopterus and *C. dewulfi*, which breed in cattle dung, are apparently influenced by the organic and clay contents of topsoil (Kettle & Lawson, 1952; Kettle, 1962b; Kremer, 1965; Zimmer *et al*., 2013a). The location of dung in pastures at an increasing distance from farms seems to influence the number of eggs laid, the hatching rate and/or larval development of these two species (Zimmer *et al*., 2014). The abundance of larvae depends on the distance from their larval microhabitat (*e.g.*, cow dung) to the farm: they are much more numerous in bovine dung localized in pastures near forests than those located in pastures near farm buildings. This situation can be due to the presence of shade at certain times of the day given by trees, preventing the dung to dry and promoting *Culicoides* larval development (Zimmer *et al*., 2014). Cattle search for shading and tend to gather near woodlands and this situation can justify an increased presence of *Culicoides* biting midges in these places, in order to perform their blood meal (Zimmer *et al*., 2014).

As coprophilic species breed in ephemeral microhabitats, so each generation needs to find new dung, these *Culicoides* species are observed near livestock, which provides both blood meal and oviposition sites. *C. chiopterus* and *C. dewulfi* are, thus, anautogenous. As woodlands generate substrates suitable for the larval development of *Culicoides* (Murray, 1957; Kremer, 1965; Glushchenko & Mirzaeva, 2008; Harrup *et al*., 2013), adult specimens will be present near this environment (Kettle, 1951; Rigot *et al*., 2013) and this situation may also influence indirectly the abundance of the coprophilic specimens present in pastures (Zimmer *et al.,* 2014). Furthermore, woodlands are involved in the circulation of some viruses (like BTV) through wild ruminants (Falconi *et al*., 2011).

Adults from *C. chiopterus* and *C. dewulfi* species can emerge in large numbers from cow dung samples from March to May and in less extant from June to October (Zimmer *et al*., 2014); they can be also found in horse dung and in old heaps of manure (Kettle & Lawson, 1952; González, López, Mullens, Baldet & Goldarazena, 2013). Zimmer, Losson, Saegerman, Haubruge & Francis (2013b) suggested that the soil under cow dung samples contain a higher proportion of *C. dewulfi* than the cow dung itself. *C. dewulfi* is adapted to a less moisture-retentive soil and rich in nutrients, whereas *C. chiopterus* prefers soil with a nutrient-retentive texture but with low organic matter fraction (Scolamacchia *et al*., 2014). Lüken *et al*., (2014) has shown that these two species were not able to survive if their breeding site is flooded, being a type D pupae (Foxi & Delrio, 2010); on the other hand, Bishop, McKenzie, Spohr & Barchia (2005) have shown that if physical characteristics of dung are altered (*e.g.*, faster drying), the development of *C. chiopterus* and *C. dewulfi* will be negatively affected.

Coprophilic *Culicoides* species are suspected to be common in farms and they are supposed to be present if cow dung is available (Cannon & Reye, 1966). However, *C. chiopterus* and *C. dewulfi* do not show an equal distribution (Nielsen, Nielsen & Chirico, 2010) and, in this way, edaphic variables significantly affect their abundance (Scolamacchia *et al*., 2014). *C.*

chiopterus and *C. dewulfi* can disperse from their larval development sites (cattle dung located in the meadow) to livestock (opened cowshed) (Zimmer, Losson, Saegerman & Haubruge, 2009).

Wittmann *et al*. (2001) have shown that areas with an annual rainfall greater than 1000 mm may not be suitable for *C. imicola* because pupae drown when breeding sites are flooded, being a type D pupae like *C. chiopterus* and *C. dewulfi* (Nevill, 1967; Foxi & Delrio, 2010).

Midges belonging to Pulicaris group have been found in a great variety of environments, particularly breeding near areas of standing water (González *et al.*, 2013), showing affinity to wet soil and marshy areas. *C. punctatus* and *C. pulicaris* were recorded near fallen leaves, river edges, forest mud and from a wet grazed field with manure (Kirkeby, Bødker, Stockmarr & Enøe, 2009), being these two species adaptable to a big variety of larval habitats (González *et al*., 2013). Since pupae from Pulicaris group can float on the water surface, the members of this group are tolerant, or might even prefer, waterlogged breeding sites (Nevill *et al*., 2007; EFSA, 2007). Habitat preferences of different *Culicoides* species are resumed in Annex 1.4.

Furthermore, more controlled experiments concerning environment (*e.g.*, soil characters, type of vegetation) and management factors (*e.g.*, manure storage) are needed to understand the ecological processes that affect *Culicoides* distribution in and around farms (Scolamacchia *et al*., 2014); even for *C. imicola*, the main vector of BTV and AHSV in Southern Europe, the Mediterranean region and Africa, the breeding ecology is not fully understood and laboratorial studies are particularly lacking (Mellor *et al*., 2000; Peters *et al*., 2014).

The availability of moisture (which is influenced by precipitation, among other factors) is the most important variable after temperature in the promotion or disruption of *Culicoides* larval development (Scolamacchia *et al*., 2014). Thus, specific physiological or behavioural adaptations of immature stages might be important key factors to explain the differences in the breeding site selection between different *Culicoides* species (Nevill *et al*., 2007).

Geographical ranges of certain vector *Culicoides* species have shown that they are less dictated by climate and more by edaphic factors, especially terrain slope and soil type. As example, *Culicoides imicola* is likely to be totally absent in places where terrain slope and soil texture induce water run-off and the rapid desiccation of the soil's surface layer, since moisture is critical for larvae survival and to complete their developmental cycle (Conte *et al*., 2007). Adult *C. imicola* individuals prefer sparsely vegetated areas in full sunlight (Conte *et al*., 2007).

1.7.3. Host detection and availability

Although some *Culicoides* species are autogenous, most of the species require a blood meal to enable egg maturation (Kettle, 1962b; Glukhova & Dubrovskaia, 1972; Mellor *et al*., 2000).

In general, most of hematophagous insects (such as mosquitoes and *Culicoides*) prefer a specific host on which they feed (Kettle, 1962b; Bartsch *et al*., 2009; Votýpka *et al*., 2009; Lassen, Nielsen & Kristensen, 2012a; Viennet *et al*., 2013), although some species are known to be generalists (Blackwell *et al*., 1994). In other way, some *Culicoides* species have opportunistic feeding behaviour concerning host distribution and density (Kettle, 1977; Garros *et al*., 2011; Lassen, Nielsen, Skovgård & Kristensen, 2011; Lassen *et al*., 2012a; Calvo *et al*., 2012).

There are several factors that may explain *Culicoides* preference for cattle instead of sheep: increased surface area, the amount of body heat produced, accessibility of blood, mechanical barriers (such as differences in the thickness of the skin), the amount of CO_2 produced and the volume and type of volatile semiochemicals exhaled (Muller & Murray, 1977; Mands *et al*., 2004; Breijo *et al*., 2014). The number of bites is essential in disease transmission, since the number of infected *Culicoides* is extremely low (Venter, Koekemoer & Paweska, 2006; Elbers *et al*., 2013) and the transmission rate increases only when the biting rate escalates.

Relative host preference rate is one of the most important parameters used for disease transmission modelling (Hartemink *et al*., 2009; Szmaragd *et al*., 2009) but it remains a neglected area of investigation (Lo Iacono, Robin, Newton, Gubbins & Wood, 2013). Several studies have shown that *Culicoides* species have a different preference for cattle and sheep hosts, in a 6:1 (Ayllón *et al*., 2014) and 9:1 (Elbers & Meiswinkel, 2014) proportions. Du Toit (1962) suggested that, when cattle and sheep are in close proximity, sheep hosts have less probability for being infected with BTV.

1.8. Laboratorial techniques for adult *Culicoides* species identification

1.8.1. Microscopic techniques

On a first approach, and for a quick identification, *Culicoides* midges can be visualized under SM and identified to species or species groups by their wing pattern (Rawlings, 1996). Specimens belonging to species groups can be further identified by COM using different identification keys (*e.g.*, Delécolle, 1985; Mathieu *et al*., 2012).

The slide mounting method is the most common for *Culicoides* preservation (Swanson, 2012). For COM, adult midges should be dissected into four parts (head, thorax, abdomen and wings), cleared with a clearing solution (*e.g.*, phenol, potassium hydroxide, warm lactic acid together with clove oil) and mounted in some medium such as Canada balsam or euparal (Swanson, 2012). Hoyer's medium can be used for both clear and mount *Culicoides* midges (Cielecka, Salamatin & Garbacewicz, 2009).

As biting midges are fixed in one plane with COM, three-dimensional structure is lost and other techniques can be used instead, like mounting different body parts into glycerine or using modern technology, like confocal laser scanning microscopy or SEM (Klaus,

Kulasekera & Schawarock, 2003; Alexandre-Pires *et al*., 2010; Swanson, 2012). SEM technique can be used to observe some differences between close related species which are very difficult or impossible to observe with SE or COM techniques. For SEM, midges must be washed (*e.g.*, ethylic alcohol), fixed (*e.g.*, glutaraldehyde in sodium cacodylate buffer), dehydrated (*e.g.*, acetone), mounted in stubs and coated (*e.g.*, gold-palladium) (Alexandre-Pires *et al*., 2010). However, SEM technique is expensive, time-consuming, laborious and requires specialized knowledge.

Morphometric tools have also been used with COM to identify more precisely different species from *Avaritia* and *Culicoides* subgenera (Pagès & Sarto I Monteys, 2005; Pagès *et al*., 2009; Augot *et al*., 2010; Muñoz-Muñoz, Talavera & Pagès, 2011), being a powerful and cheap characterization tool for these biting midges (Hajd Hanni *et al*., 2014). However, some different sized specimens belonging to the same species can occur, since *Culicoides* size is influenced by different environmental factors such as temperature (Birdsall, Zimmerman, Teeter & Gibson, 2000; Smith & Mullens, 2003) food concentration or larval density (Jirakanjanakit *et al*., 2007) and, for this reason, some entomologists have suggested that size is not always taxonomically useful (Lane, 1981a,b), although it is also influenced by genetic effects (Hajd Hanni *et al*., 2014). Several studies have shown that other morphological characters have also phenotypic variations within species. Furthermore, these techniques require slide mounting of specimens, which is very time-consuming and laborious (Delécolle, 1985; Pagès & Sarto I Monteys, 2005; Augot *et al*., 2010).

1.8.2. Molecular biology techniques

Molecular biology is another useful tool for species identification inside *Culicoides* groups and complexes, just as it happens with other invertebrate species (Gomes *et al*., 2012, for the example of *Culex pipiens* complex). As example, inside Obsoletus group there are 5 different species present in Western Europe that are very difficult to distinguish between each other, even by well-trained scientists (Meiswinkel *et al.*, 2004a; Pagès & Sarto I Monteys, 2005; Balczun, Vorsprach, Meiser & Schaub, 2009; Garros *et al*., 2010).

There are several molecular markers that can be used for phylogenetic analysis within *Culicoides* genus (Harrup *et al*., 2014). According to their genetic region, they can be grouped as: (1) mitochondrial (COI, COII, 28S rRNA, 18S rRNA, 16S rRNA and Cyt*b*), (2) ribosomal (ITS1 and ITS2) and (3) nuclear (CAD). Animal mitochondrial genome is a better target for analysis than nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Herbert *et al*., 2003) from mother to offspring.

Several molecular methods are available and supply unambiguous results: sequencing of the nuclear internal transcribed spacer (ITS1 and ITS2) (Cêtre-Sossah *et al*., 2004) or the mitochondrial cytochrome oxidase subunit I (COI) (Nolan *et al*., 2007) can be performed to

identify doubtful specimens by polymerase chain reaction (PCR) and its variants (multiplex PCR and qPCR), being the most commonly markers used.

Ribosomal deoxyribonucleic acid (rDNA) markers have been used to investigate phylogenies (ITS1 and ITS2: Gomulski *et al*., 2005; Gomulski *et al*., 2006; Perrin *et al*., 2006; 28S: Hajd Henni *et al*., 2014) interspecific genetic distances (ITS1: Nielsen & Kristensen, 2011) and population structure (ITS1: Ritchie, Blackwell, Malloch & Fenton, 2004) within *Culicoides*. ITS regions polymorphism has been used to develop both conventional (Mathieu *et al*., 2007; Stephan, Clausen, Bauer & Steuber, 2009) and qPCR (Cêtre-Sossah *et al*., 2008; Monaco, Benedetto, di Marcello, Leilli & Goffredo, 2010; Mathieu *et al*., 2011) assays for identification of BTV vector species from individual and pooled samples.

COI is a mitochondrial DNA (mtDNA) marker that exhibit a high level of interspecific polymorphism and few differences within species (intraspecific polymorphism) (Hebert, Cywinska, Ball & deWaard, 2003), being a useful marker for phylogenetic studies (Harrup *et al*., 2014) and as a "DNA barcoding" system of animal life (Herbert *et al*., 2003). The use of DNA barcoding permits species discrimination both easier and faster, and thus more cost-efficiently. These molecular assays are currently used to studies related to host-vector contact, larval ecology, vector competence, insecticide susceptibility, seasonal dynamics and spatial distribution (Garros *et al*., 2014). There is a good evidence showing that COI barcode region is suitable for discrimination of species of *Avaritia* (Linton *et al.,* 2002; Jan Debila, 2010; Ander, Troell & Chirico, 2013; Bellis *et al*., 2014) and *Culicoides* (Lassen, Nielsen, Skovgård & Kristensen, 2012b; Ander *et al*., 2013) subgenus; however there are indications that variation in this region is not enough for species delimitation inside some subgenera, like *Beltranmyia* (Ander *et al*., 2013; Bellis, 2013).

Multiplex PCR for *Culicoides* species identification can be used to investigate the abundance, distribution and bionomics of these biting midges (Harrup *et al*., 2013; Viennet *et al*., 2013). However, molecular approach implies financial support, time, accurate materials and specific laboratorial conditions (Augot *et al.,* 2010). The formation of non-specific bands, primer dimers or weak signals caused by inhibiting interactions between primers are the most common problems of multiplex PCR (Mathieu *et al*., 2007). Furthermore, these techniques can only identify small numbers of species simultaneously (Deblauwe *et al*., 2012). A major uncertainty in *Culicoides* identification by multiplex assays is the potential presence of cryptic undescribed species within the European fauna which may cross-react with detection systems for known species. Although some of those species are described in literature, they are not frequently investigated (Pagès *et al*., 2009).

Quantitative PCR is a highly cost-effective technique, allowing the processing of one hundred *Culicoides* individuals in a single extraction by identification of pool specimens (Garros *et al*., 2014; Harrup *et al*., 2014) based on ITS and COI polymorphism analysis (Cêtre-Sossah *et al*., 2008; Monaco *et al*., 2010; Mathieu *et al*., 2011; Wenk *et al*., 2012)

DNA microarray, a technique based on probe hybridization, can be also used for species identification. Many species can be analysed at a time with high sensitivity and specificity and low background signal noise; however it is an expensive technique (Zhou & Thompson, 2004).

Phylogenetic studies were initially performed through analysis of single genes or small numbers of genes. However, it is recently entering into the use of genome-scale datasets (Stencel & Crespi, 2013) to infer evolutionary relationships (Brito & Edwards, 2009; Xi *et al*., 2012; Barrett, Davis, Leebens-Mack, Conran & Stevenson, 2013; Narum, Buerkle, Davey, Miller & Hohenlohe, 2013), in the so called "phylogenomics" (Delsuc, Brinkmann & Philippe, 2005). The entire genome dataset of *Culicoides sonorensis* Wirth & Jones, 1957, was produced in 2013 (Fife, Carpenter, Mertens & Kersey, 2013; Nayduch *et al*., 2014), representing a vital step to additional *Culicoides* species genome analysis and by which they can be assembled, annotated and incorporated into future phylogenomic studies (Harrup *et al*., 2014).

Only a few studies correlate phylogenetic clades with detailed morphological analysis (Bellis, Dyce, Gopurenko & Mitchell, 2013); however, high-quality phylogenetic analysis should be performed together with traditional morphological taxonomy (Harrup *et al*., 2014) in a scientific area called "integrative taxonomy" (Will, Mishler & Wheeler, 2005; Padial, Miralles, De la Riva & Vences, 2010; Schlick-Steiner *et al*., 2010). Integrative taxonomy gives important information about *Culicoides* subgeneric classification and identification of new species (Bellis *et al*., 2013; Bellis *et al*., 2014) and can help to resolve species complexes, as well as investigate phenotypic plasticity (Harrup *et al*., 2014, Alström *et al*., 2015).

1.9. *Culicoides* biting midges control

Together with vaccines (when and where they are available), the use of insect repellents and/or insecticides have to take part of integrated control programmes against diseases transmitted by *Culicoides* (Capenter, Mellor & Torr, 2008c). There are several mechanisms to control *Culicoides* biting midges: (1) insecticide application in larval and adult habitats; (2) environmental interventions to eliminate larval development sites; (3) control methods of adult resting sites, like stables, or directly on hosts with insecticides; (4) use of repellents or attractants to kill adult biting midges; (5) housing livestock in mesh screened buildings when *Culicoides* activity is maximum; (6) *Culicoides* capture with traps and (7) biological control strategies (EFSA, 2008; de Heredia & Lafuente, 2011). Environmental contaminants might also be manipulated for control of *Culicoides* (Mullens, McDermott & Gerry, 2015).

1.9.1. *Culicoides* control in larval and adult phases

Larval control is sometimes difficult because of the extensive and dispersed larval habitats (Papadopoulos, Bartram, Carpenter, Mellor & Wall, 2009). Larvicides have not been effective

to reduce *Culicoides* populations and, besides, there are several environmental restrictions to the use of chemical agents in open spaces (de Heredia & Lafuente, 2011). Historically, the first control was made with organochlorides (DDT) as well as with pyrethrins and pyrethroids; then, they were substituted by organophosphates, although recent studies have shown that organophosphates are not so effective in killing *Culicoides* biting midges as pyrethroids (Venail *et al*., 2015). Pyrethroids cannot be used as larvicides due to their toxicity to the invertebrate aquatic fauna (Carpenter *et al*., 2008c).

The control of adult *Culicoides* is sometimes hard to achieve because adult biting midges spend limited time feeding on their hosts in comparison to many other ectoparasite groups (Papadopoulos *et al*., 2009). Moreover, due to their small size, *Culicoides* can enter through conventional nets used against other biting insects, such as mosquitoes. These nets do not prevent their entry, restrict air flow (Porter, 1959) and apparently increases *Culicoides* population inside the net (Calvete *et al*., 2007), since it not allows sufficient contact of biting midges with the insecticide (Del Río, Barceló, Lucientes & Miranda, 2014). Only by increasing shelter in open areas using dense and impenetrable physical barriers together with insecticide treatment of those same barriers has a degree of protection against biting midges (Calvete *et al*., 2010). For a higher degree of protection, livestock insecticide treatment should be performed together with treated barriers (Mullens, Velten, Gerry, Braverman & Endris, 2000; Mullens, Gerry & Velten, 2001; Doherty, Johnson & Reid, 2001; Mehlhorn, Schmahl, D'Haese & Schumacher, 2008a). However, welfare concerns associated with airflow reduction and animal confinement restricts the use of completely enclosed structures for housing livestock species (Calvete *et al*., 2010).

1.9.2. Chemical control

1.9.2.1. Insecticides

A wide range of compounds are used to control *Culicoides* in order to avoid their blood meal in hosts. These products can be divided into: (1) pour-on formulations (usually applied along the animal back-line); (2) ear tags attached to animals; (3) dipping formulations (where the animal is immersed in the product) or (4) injected systemic formulations. The first three methods are based in pyrethrins (natural insecticides from *Chrysanthenum cineriaefolium* and similar plants) and synthetic pyrethroids (*e.g.*, permethrin, deltamethrin, cypermethrin, flumethrin, cyfluthrin, cyhalothrin) or organophosphates, although other active ingredients may be used (*e.g.*, macrocyclic lactones) (EFSA, 2008; de Heredia & Lafuente, 2011).

Injectable systemic compounds are commonly based in macrocyclic lactones (*e.g.*, avermectins, ivermectins) and toxicity occurs after ingestion of a blood meal containing the active ingredient (Hansen, Stemme, Villar & Buck, 1994; EFSA, 2008). However, systemic compounds have several drawbacks by not being useable in dairy herds and possessing long withdrawal times for meat. Moreover, these active substances are only effective in

dosages that are not permitted by legislation and they are becoming a real concern in some species because of the development of anthelminthic resistances. Furthermore, there are concerns regarding the environmental impact of these products upon non-target fauna (EFSA, 2008; de Heredia & Lafuente, 2011).

Pyrethroids insecticides have the potential to provide effective and convenient, although short-term, local control, by preventing onwards transmission from viraemic animals (Papadopoulos *et al*., 2009). Since different *Culicoides* species have different predilection sites for feeding, such as back (*C. punctatus* and *C. nubeculosus*) (Townley, Baker & Quinn, 1984; Braverman, 1991), belly (*C. lupicaris*) (Jones & Akey, 1977; Townley *et al*., 1984) or lower legs (*C. chiopterus*, *C. dewulfi* and *C. obsoletus*) (Townley *et al*., 1984; Papadopoulos *et al.*, 2009), insecticides may have different degrees of efficiency.

Pyrethroids can be classified as Type 1 (*e.g.*, permethrin) or 2 (*e.g.*, deltamethrin), being the former less toxic than the second (Venail *et al*., 2015). These active substances are extremely selective for insects over mammals since insect sodium channels can be as much as 100 times more sensitive than mammalian brain sodium channels (Warmke *et al*., 1997). Briefly, they cause the sodium channel to remain open during nervous stimuli. This causes the now modified channel to stabilize in a hyperexcitable state. Type 2 pyrethroids keep the sodium channel open for a significantly longer period than Type 1 pyrethroids, which in part explains the differential toxicity between these two classes. Another classification is made with Type I compounds (*e.g.*, permethrin) causing rapid onset of hyperactivity and repetitive action potentials and Type II compounds (*e.g.*, cypermethrin) causing lethal effects in very low doses (Baynes, 2009).

Tests using permethrin or cypermethrin as ear tags resulted in shorter periods of protection, possibly because they do not reach in sufficient amounts the farthest body areas such as feet; however, the protection is longer with two ear tags than with one (Liebisch & Liebisch, 2008; Liebisch, Liebisch, Heine, Thienel & Henrichs, 2008).

Papadopoulos *et al*., (2009) have shown that alpha-cypermethrin applied as pour-on kill a high proportion of *C. nubeculosus* when in contact with sheep wool or cattle hair treated with this active ingredient, for at least 21 days after treatment, independently of the body part or the host species. Page, Labuschagne, Venter, Schoeman & Guthrie (2014) have shown that high-density polyethylene (HDPE) mesh treated with alpha-cypermethrin and applied to stables or jet stalls could be used to effectively protect horses against *Culicoides*, mainly *C. imicola*.

Cyhalothrin was tested in laboratory and showed to be effective against *C. imicola* (Braverman, Chizov-Ginzburg, Pener & Wilamowski, 2004); however, lambda-cyhalothrin was found to be nearly 10 times more efficient than cyhalothrin against *C. imicola* (Braverman, Wilamowski & Chizov-Ginzburg, 1995), showing that this species control can be achieved with a much smaller quantity of insecticide and would be both environmentally and

economically beneficial (Braverman *et al*., 2004). Nevertheless, these two molecules do not act as repellent, since *C. imicola* lands in filter paper impregnated with them (Braverman *et al*., 2004). In Australia, the use of ivermectin as a systemic insecticide causes 99% of mortality in *Culicoides brevitarsis* 10 days following treatment (Braverman, 1994). Furthermore, the dung of ivermectin-treated cattle is also larvicide for *Culicoides* spp. for up to 28 days (Webster, Gard, St. George & Kirkland, 1991).

1.9.2.2. Repellents and attractants

Insect-repelling compounds can be used to repel *Culicoides* from potential hosts, thereby avoiding attack. Attractive compounds can be used as bait to attract biting insects (Logan & Birkett, 2007).

Myrica gale (Myricaceae) is a small shrub, whose droplets are composed by a complex mixture of terpenoids (Lawrence & Weaver, 1974), being a deterrent for *C. impunctatus* to get close to a host (Stuart, 1990). Furthermore, Blackwell, Evans, Strang & Cole (2004) evaluated the efficacy of neem oil against this species, showing significant repellence for a 1% concentration and significant reduction in blood feeding on neem treated membranes.

Sollai, Solari, Masala, Crnjar & Liscia (2007) have shown that antihelmintics (doramectin and ivermectin) have an effect on the olfactory sensitivity of *C. imicola* towards the animal host by reducing the response to compounds that attract the insect and consequently reducing the possibility of bites on sheep.

In South Africa, 15% *N,N*-diethyl-3-methybenzamide (DEET) has a significant repellent effect against *Culicoides* species, including *C. imicola*, when applied in horses and their stable environment, along with stabling and meshing of stables (Page, Labuschagne, Nurton, Venter & Guthrie, 2009) and it remains the gold standard of currently available insect repellents (Fradin, 1998). Other compounds, some of them with insecticide properties, previously evaluated via repellent-impregnated mesh are reported in Table 1.4.

Table 1.4. – Repellent compounds previously evaluated on impregnated meshes.

Compounds	Commercial name	References
Meliaceae-derived plant extract	Ag 1000	Braverman *et al*., 1998, 1999, 2000
Oregano	-	
Mixture of plant extracts: oils of sage, rosemary and oregano	Herbipet	
Cypermethrin and other pyrethrins	Tri-Tec14	
Type II pyrethroid containing α-cyano,3-phenoxybenzyl moiety	Pyrethroid-T	
Permethrin	Stomoxin	
Eucalyptus extract containing p-methane-3,8-diol, with isopulegol and citronellol	Mosi-guard	
Plant extracts	Lice Free	

Romón *et al*. (2012) tested repellent substances in CDC traps on field proofs and *C. obsoletus/C. scoticus* were significantly repelled by neem oil, DEET and allyl isothiocyanate. However, *C. lupicaris* was not repelled by DEET, but by neem oil and allyl isothiocyanate. *C. punctatus* did not show repellence to any of the three above mentioned compounds.

A mixture of organic fatty acids that are normal constituents of vertebrate skin (octanoic, nonanoic and decanoic acids) have a significant repellent effect against *Culicoides* species and especially *C. imicola* when applied to polyester mesh and tested with suction light traps (Venter *et al*., 2011).

The results obtained with citronella, a natural oil, have been different between several authors (Martínez-de la Puente *et al*., 2009; Page *et al*., 2009). Venail (2015) refer that geranium and citronella essential oils have a repellent effect equivalent to the DEET and a lethal effect equivalent to that of deltamethrin. Although they could be used instead of insecticides to reduce pressure and so decrease the risk of development of resistance to insecticides, their persistence are very short and their use in animals is unauthorized (Venail, 2015).

Besides DEET and permethrin, picaridine has shown good results as insect repellent (Venail, 2015), although their efficiency is limited in time and regular applications are necessary (Carpenter *et al*., 2005). Eucalyptus and lavender have also shown a repulsive effect against *Culicoides* (González, Venter, López, Iturrondobeitia & Goldarazena, 2014).

Modern traps can be equipped with sophisticated mechanisms of attraction, like heat, steam, octenol/phenol mixtures, CO_2 and lactic acid, among others, which allows a reduction in mosquito population, although a significant reduction in bite rates and virus transmission has not yet been achieved (de Heredia & Lafuente, 2011).

1.9.3. Biological control

Biological control is an environmentally sound and effective means of reducing or mitigating pests populations, permanently or temporarily, through the use of natural enemies (parasitoids, predators, pathogens, antagonist or competitor populations) (van Driesche & Bellows, 1996; van Driesche, Hoddle & Center, 2008).

1.9.3.1. Biocontrol agents

The larval stage of the trombidioid mites *Centrotrombidium blackwellae* (Baker, 1999) and *Centrotrombidium culicoides* (Vercammen-Grandjean, 1957) have been found parasitizing *C. impunctatus* (Baker, 1999), *C. circumscriptus*, *C. maritimus* and *C. pulicaris* (Vercammen-Grandjean, 1957; Vercammen-Grandjean & Feider, 1973).

Concerning nematodes, there are a total of 8 different genus that can parasitize *Culicoides*, being mermithids the major nematode group that uses biting midges as definitive hosts (Table 1.5.).

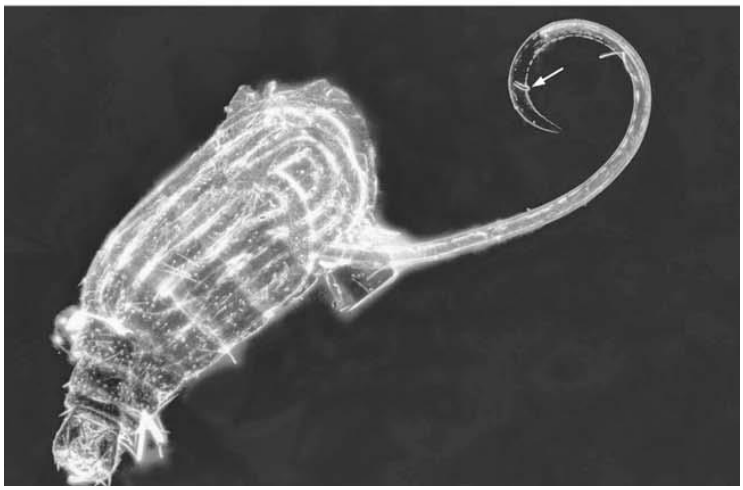
Table 1.5. – Nematodes found parasitizing *Culicoides* biting midges.

Nematode Family	**Nematode genus/species**[1]	***Culicoides* species**	**References**
Tetradonomatidae	*Aproctonema chapmani*	*C. arboricola*	Rubzov, 1967 Nickle, 1969 Gafurov *et al*., 1984 Luhring *et al*., 1997 Sarto i Monteys *et al*., 2003 Mullens *et al*., 2008 Poinar *et al*., 2008 Bradley *et al*., 2010 Mullens *et al*., 2010
Mermithidae	*Agamomermis helis*	*C. pulicaris*	
	Ceratomermis	*Culicoides* spp.	
	Gastromermis		
	Heleidomermis cataloniensis	*C. circumscriptus* *C. parroti*	
	Heleidomermis magnapapula[2]	*C. sonorensis*	
	Heleidomermis magnapulata	*Culicoides* spp.	
	Heleidomermis vivipara	*C. stigma* *C. nubeculosus* *C. puncticollis* *C. circumscriptus*	
	Limnomermis	*Culicoides* spp.	
	Romanomermis		
	Spiculimermis		

[1]Parasites found in *Culicoides* larval, pupal and/or adult stages; [2]This parasite can reduce the emergence of *Culicoides sonorensis* in 84%.

Heleidomermis nematodes are parasites of *Culicoides* larval phases, emerging in the stage IV, although, in some situations, they can exist in pupal and adult phases, being a common find in this last phase (Mullens *et al*., 2010) (Figures 1.28. and 1.29.).

Figure 1.28. – A male C. circumscriptus parasitized with H. cataloniensis (adapted from Mullens, Sarto i Monteys & Przhiboro, 2008)



White arrow: male nematode spicule.

Figure 1.29. – A female *C. parroti* parasitized with *H. cataloniensis* (adapted from Mullens *et al*., 2008)

It is also known that nematodes from *Heleidomermis* genus invariably kill their hosts upon emergence and so the percentage of parasitism found in *Culicoides* population is representative of insect mortality (Sarto i Monteys & Saiz-Ardanaz, 2003).

Virus from Iridoviridae family have been isolated from some *Culicoides* species, although some laboratorial tests have shown that infection rate is too low (<1%) and the virus rarely infects the larva satisfactorily when put together in laboratory (de Heredia & Lafuente, 2011). Furthermore, bacteria (*e.g.*, *Bacillus thuringiensis*), as a biocontrol agents, have not given the expected laboratorial results (Carpenter e*t al*., 2008c).

Culicinomyces clavisporus is a pathogenic fungus which has a larvicide action in *C. nubeculosus* (Unkles, Marriott, Kinghorn, Panter & Blackwell, 2004). Ansari, Carpenter & Butt (2010) and Ansari, Pope, Carpenter, Scholte & Butt (2011) have shown that *Metarhizium anisopliae* is effective in killing larvae and adult phases of *C. nubeculosus*, while Nicholas & McCorkell observed that this fungus was able to infect and kill adult *C. brevitarsis* over an eight-day continuous exposure period (Figure 1.30.) Despite all this, field studies with entomopathogenic fungi are still missing (Lüken *et al*., 2014).

Figure 1.30. – *C. brevitarsis* adults uninfected (A) and infected (B) with *M. anisopliae* (original from Nicholas & McCorkell, 2014).

Lagenidium giganteum is a microbial parasite which belongs to a group of organisms that, although they look like fungi and have a “fungal lifestyle”, nonetheless are related to diatoms and red algae (Cavalier-Smith, 1986; Barr, 1992; Kerwin, 2007). This species have been isolated from *Culicoides* in Australia, with infection rates varying between 1-33% (Wright & Easton, 1996). Other fungi found in *Culicoides* belong to *Nosema* and *Vavraria* genus but in small rates of infection (de Heredia & Lafuente, 2011). These organisms are Microsporidia, unicellular parasites and have been recently reclassified as fungus.

Culicoides biting midges can be also predated by ceratopogonids species from *Bezzia* and *Probezzia* genus, which attack *Culicoides* species in laboratorial conditions, and by some insects, like *Tachydromia minuta*, larvae of Tipulidae, Chironomidae and Culicidae families and adults from Formicidae family, which have been observed feeding in *Culicoides* (de Heredia & Lafuente, 2011). Two species of predatory beetles were also identified: *Cicindela suturalis*, a tiger beetle, feeding of *C. phlebotomus* nymphs, and *Elaphrus cupreus*, which can devour up to 60 *C. riethi* pupae in half an hour (Rieb & Delécolle, 1981).

Finally, a small study by Reeves (2010) showed that in the presence of a common freshwater predator named *Hydra littoralis* (Phylum Cnidaria, Class Hydrozoa), *C. sonorensis* larvae spend more time actively swimming than they do in the absence of a predator, indicating a possible avoidance behaviour that might cost larvae considerable energy and reduce survival.

1.9.4. Biotechnological control

Biotechnological control can be defined as controlled and deliberate manipulations of biological systems (*e.g.*, DNA-based technologies, bioinsecticides, hormones) to achieve efficient insect pest control.

1.9.4.1. Hormones

Insect growth regulators (IGRs) gained huge importance in the 1980s and 1990s, since they are not pesticides and are harmless to pets, livestock and humans. However, they only affect the following insect developing stages: eggs, larvae and pupae. Due to this situation, their effective control is only achieved several weeks after treatment. To be more effective, they are usually commercialized together with adulticides. There are two types of IGRs: juvenile hormone analogues (JHAs) and insect development inhibitors (IDIs) (Baynes, 2009; de Heredia & Lafuente, 2011)

JHAs maintain insects in their egg or larval stages, not allowing them to grow into adult stages. The main JHAs include methoprene, pyriproxifen, fenoxycarb and cyromazine, being the former two JHAs the most frequently used in veterinary medicine. They are usually commercialized with adulticides such as pyrethrins and/or pyrethroids (Baynes, 2009).

IDIs, such as the benzoylphenyl urea compounds, interfere with the insect exoskeleton development by inhibiting chitin synthesis or deposition pathways (Baynes, 2009).

Chapter 2: Morphological and molecular study of different *Culicoides* species in Portugal

2.1. Introduction

In 2005, a year after the BTD outbreak in mainland Portugal, and in recognition of the high and continuing threat to the Portuguese livestock sector from *Culicoides*-borne viruses, the Portuguese authorities (Direção Geral de Veterinária [DGV] in partnership with the Faculty of Veterinary Medicine) established a National Entomologic Surveillance Program (NESP) for BTD. The NESP covered all regions of the country, including mainland Portugal, Azores islands (São Miguel, Santa Maria, Terceira, Graciosa, São Jorge, Pico, Faial, Flores and Corvo) and Madeira islands (Madeira and Porto Santo). As BTV was absent from Azores and Madeira archipelagos, the programme in the islands was carried out with different design, using less frequent sampling (Ribeiro *et al*., 2015).

During NESP, which operated from 2005 to 2013, *Culicoides* midges were captured in different cattle, sheep and goat farms. The NESP was created to know the distribution of different *Culicoides* species, reporting to the DGV the presence of *Culicoides* vectors of BTD, for they could act in real time and prevent the spread of the disease in case of an outbreak. This information was obtained by the collection and identification of *Culicoides* species in Portugal, mapping their distribution and quantifying their abundance with outbreaks of *Culicoides*-transmitted diseases (Ribeiro *et al*., 2015). Although the NESP lowered the activity since August 2010, collection of midges maintained until February 2013.

Similarly to what happened in other Entomologic Surveillance Programs in several European countries, a quick identification of *Culicoides* species by their wing pattern and evaluation of female parity status allowed the evaluation of epidemiologic risk of BTD transmission in Portugal.

Furthermore, animal movement control and mass vaccination of the animals were other of the procedures taken (Rodrigues, 2008). Bluetongue outbreaks occurred continuously in Portugal from 2006 to 2013 (OIE – World Animal Health Information Database [WAHID], 2014).

Additionally, from April to November, 2015, new captures started to be performed in the scope of the VectorNet European network (OC/EFSA/AHAW/2013/02) in mainland Portugal and other European countries with the main objective to compare *Culicoides* species that were captured near cattle and horse farms.

2.2. Objectives

The aims of this work were:

1) Identify the different *Culicoides* species present in mainland Portugal and Azores and Madeira archipelagos captured during the NESP (2005-2013) and VectorNet European network (2015), by conventional microscopy (SM and COM), SEM and molecular biology, as well as their seasonality in mainland Portugal.

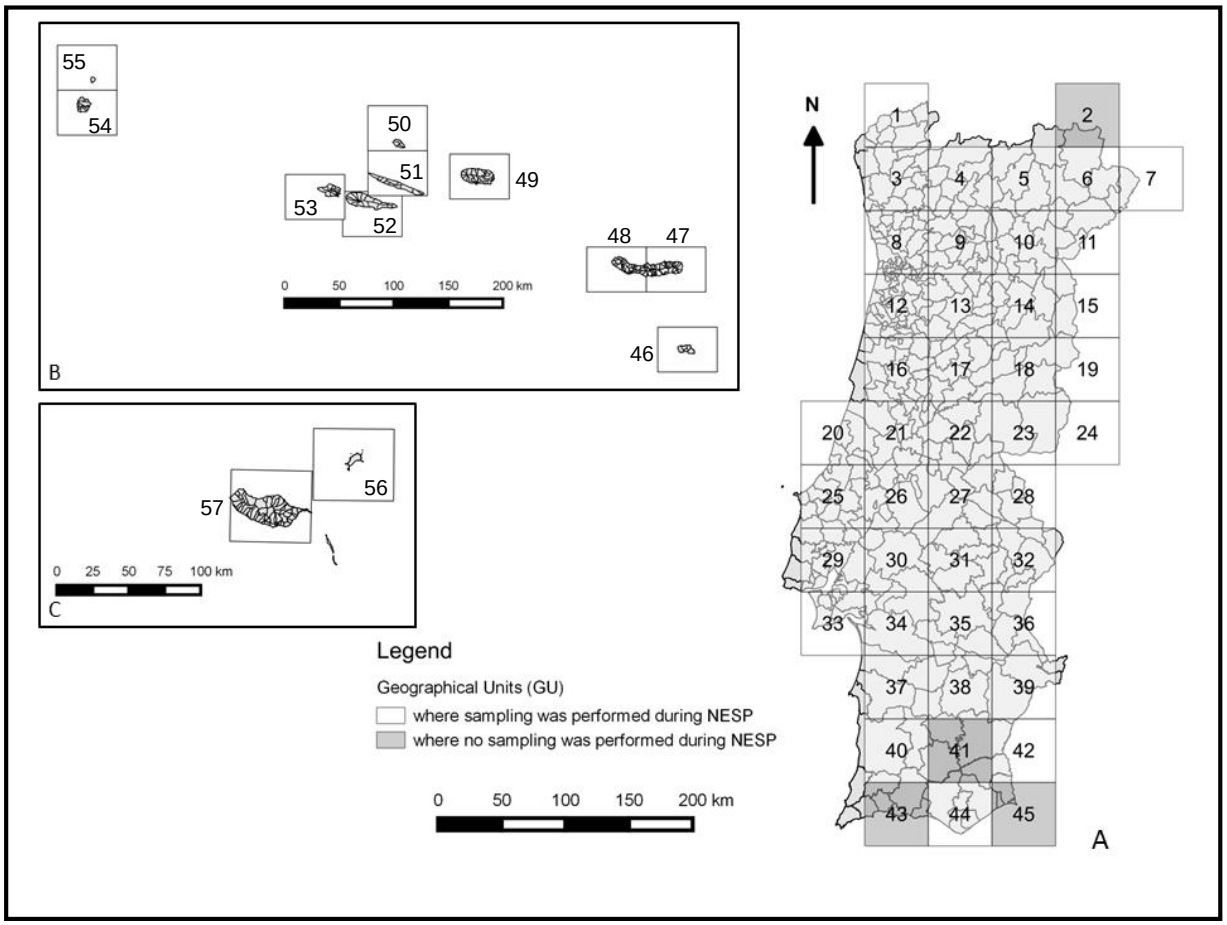
2) Recognize specimens belonging to an unknown species (temporarily named as "*C. sublupicaris*") and additional studies using COM, SEM and molecular biology techniques, in an integrative taxonomy approach.
3) Summarize available information about *Culicoides* genus in Portugal since 1956, by an exhaustive bibliographic research.
4) Compare *Culicoides* species captured in cattle and horse farms.
5) Elaboration of an identification key for the Portuguese *Culicoides* fauna.

2.3. Materials and methods

2.3.1. The NESP and VectorNet European network

To ensure systematic coverage during NESP, Portugal area was divided into 57 squares, each one with 50 km of side, named geographical units (GUs) (Figure 2.1.).

Figure 2.1. – Geographical Units in mainland Portugal and in Azores and Madeira archipelagos (original by Madeira, S.).



A – Mainland Portugal; B – Azores archipelago; C – Madeira archipelago. Azores archipelago GUs: 46 – Santa Maria island; 47 and 48 – São Miguel island; 49 – Terceira island; 50 – Graciosa island; 51 – São Jorge island; 52 – Pico island; 53 – Faial island; 54 – Flores island; 55 – Corvo island. Madeira archipelago GUs: 56 – Porto Santo island; 57 – Madeira island.

Four mainland Portugal GUs were not sampled (2, 41, 43 and 45) since they were not considered to be of epidemiological interest due to low livestock densities.

Within each GU, two suitable farms were identified using the national database of farms and farmers and farmers were contacted by the National Authority for Animal Health (DGV) and asked if they agreed to participate in the national programme for BTD control. All the farmers gave permission to place the traps in their farms, as it was explained that it would be a good contribution for the protection of animal health.

For an easy localization of the selected farms, the Global Positioning System (GPS) was used. This information can be further used to localize farms within the Nomenclature of Territorial Units for Statistics, subdivision 3 (NUTS III), with 25 subregions in which Portugal can be divided (Annex 2.1.)

Eligible farms should possess a minimum of five horses or ruminants (preferably cattle), be localized at least 2.5 km from the coast line and separated by at least 10 km from each other. The use of insecticides in these farms was not allowed during the NESP.

The presence of livestock species and bodies of water on the farm, human housing and other livestock farms within 10 km was recorded by state veterinary staff during trap placement, by completion of a standardized questionnaire.

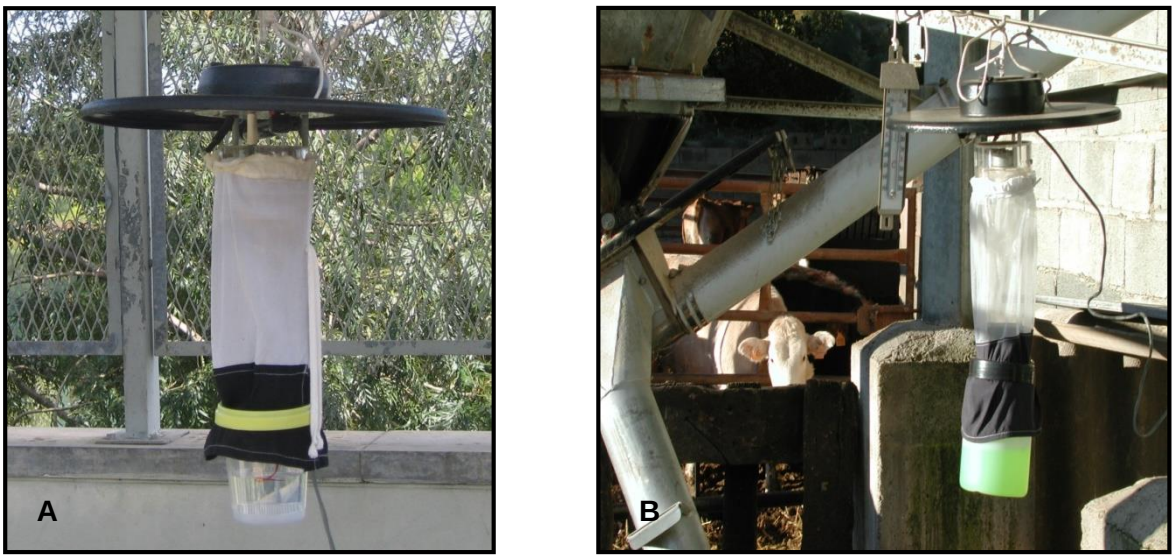
Land cover types in the same parish were extracted from the cartographic farming database maintained by the Institute for Agricultural Financing of the Portuguese Ministry of Agriculture. The proximity of water bodies, human housing, other farms and vegetation to the trapping site was later verified using aerial photography.

For VectorNet European network, two near farms (one with cattle and another with horses) were selected in two different districts of mainland Portugal (Leiria and Castelo Branco) to compare *Culicoides* species that were present near these animal species.

2.3.2. Insect sampling

In the NESP, *Culicoides* were collected using miniature CDC light traps (CDC miniature blacklight model 1212, John Hock, USA) fitted with 4 W UV bulbs, suction fans and LCS-2 Photoswitch systems (Figure 2.2.).

Figure 2.2. – Miniature CDC light trap (A) placed within 30 m of animal enclosures, 1.70 m above ground (B).

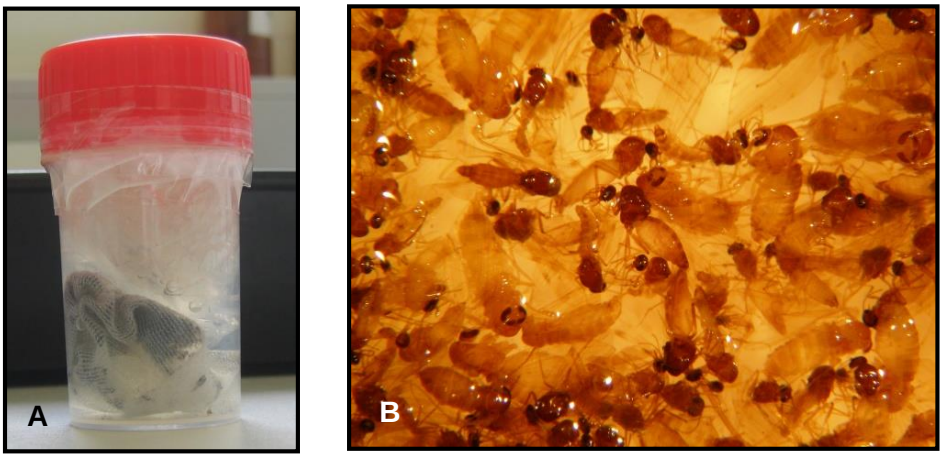


A – Original photo; B – Photo taken during the NESP, 2006.

CDC light traps were placed within 30 m of animal enclosures, 1.70 m above ground (Figure 2.2.). The LCS-2 Photoswitch system automatically switched the trap on at dusk and off after dawn. In mainland Portugal, each trap operated for one night per week throughout the year. In Madeira and Azores archipelagos, each trap operated for two nights, once every six months.

Insects were collected into flasks containing 75% of 70° ethanol and 25% of ethylene glycol as antifreeze, in a final volume of 500 ml, and were brought to the Laboratory of Parasitology and Parasitic Diseases at the Interdisciplinary Centre of Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa) (Figure 2.3.). When field collections had an estimated number between 2.000 and 5.000 insects and more than 5.000 insects, 25% and 2.5% dilutions were performed, respectively.

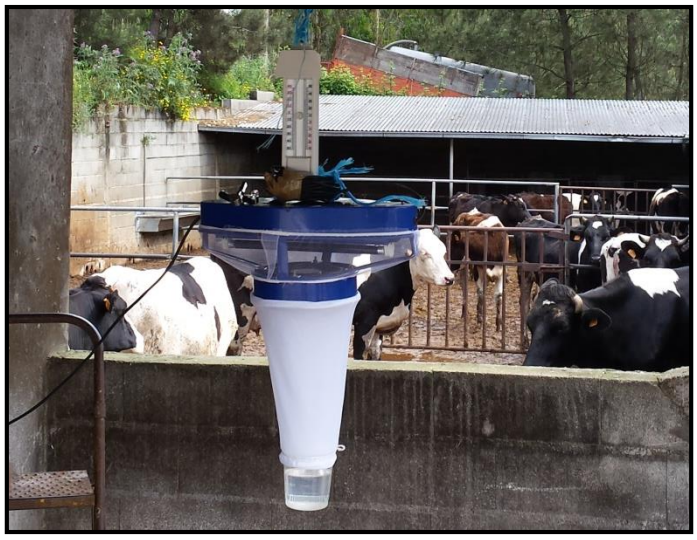
Figure 2.3. – Flask containing a performed captured (A) and sample analysis with SM (B).



Original photos.

For VectorNet European network, down-draught 220 V Onderstepoort light traps were used (Figure 2.4.).

Figure 2.4. – Onderstepoort light trap (original by Madeira, S.)



Onderstepoort light traps were placed within 30 m of animal enclosures, 1.70 m above ground. Each trap operated for one night per month from April to November, 2015. Methods for insect collection and performed dilutions were the same as pointed out for the NESP.

2.3.3. Morphological and molecular identification of *Culicoides* species

2.3.3.1. Stereoscope and composed optical microscopy

Morphological identification of female *Culicoides* biting midges was performed in Laboratory of Parasitology and Parasitic Diseases at the CIISA, FMV, ULisboa. *Culicoides* specimens were evaluated with SM (Olympus SZ51) and identified to species by their wing pattern or, when their identification was not possible by this mean, they were dissected into different body parts (head, thorax, abdomen, wings and legs) using 26 Gauge (0.404mm diameter) needles, followed by mounting in glass slides covered with a coverslip using Hoyer's medium (Annex 2.2.) and dried in an incubator at 37 °C for 3-4 days. These specimens were then examined using COM (Olympus BX50 microscope) and different body structures (eyes, cibarium, palpus, antennal flagellomeres, mouthparts, legs and wings in both sexes, aedeagus, parameres, styli, tergites, lamellae and basal membrane in males, and spermathecae in females) were characterized and measured to identify specimens to species. Photographs of different species were obtained with an Olympus DP10 camera.

2.3.3.2. "*Culicoides sublupicaris*" specimens

Within *Culicoides* spp., a group of specimens with a wing pattern different from those reported in existing literature and morphologically similar to *C. lupicaris* and *C. newsteadi*

were temporarily classified as "*Culicoides sublupicaris*" (Delécolle, personal communication, November 15, 2010). For these specimens, an additional study with SEM and molecular biology was performed.

For SEM:

Two specimens of "*C. sublupicaris*", *C. lupicaris* and *C. newsteadi* were washed in ethylic alcohol at 70° and fixed in 2.2% glutaraldehyde (Panreac, ref. 163857.1611) in sodium cacodylate buffer (Sigma Aldrich, ref. 70114). Then, dehydration was performed with crescent concentrations of acetone (from 70% to 100%). Specimens were dried using critical point drying method (JOEL fine coater JFC-1200), mounted in stubs and coated with gold-palladium (Sigma Aldrich, ref. 716928) and observed in a electronic microscope (JEOL-JSM-5200LV), with accelerating voltage ranging from 10 to 25 kV (Alexandre-Pires *et al*., 2010).

For molecular biology:

Single midge total DNA of "*C. sublupicaris*" (n=31), *C. punctatus* (n=26), *C. lupicaris* (n=15), *C. newsteadi* (n=5) and *C. pulicaris* (n=21) specimens was extracted using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Crawley, UK) following manufacturer's instructions, with a final elution volume of 100 µl. Sequences of mtDNA COI gene were amplified by PCR in a final volume of 50 µl. Each reaction used 2 mM $MgCl_2$, 1 mM dNTPs, 0.2 µM of each primer, 1 polymerase unit and 2 µl of genomic DNA. Forward and reverse primers used were respectively C1-J-1718 and C1-N-2191 (Simon *et al*., 1994). The amplification program took place in a GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA thermocycler, consisting of an initial denaturing step at 94 °C for 3 min, followed by 35 cycles at 94 °C, 30 s; 50 °C, 30 s; 72 °C, 30 s and a final extension step at 72 °C for 7 min.

For genetic sequence analysis:

PCR products were sequenced on both strands by Cogenics (France). The obtained COI sequences were aligned using BioEdit sequence alignment editor software (version 5.0.9 for Windows; Hall, 1999) with ClustalW algorithm. The Maximum Likelihood (ML) analysis were carried out with MEGA version 5, incorporating best fit models of sequence evolution determined using the Akaike Information Criterion (AIC) and employing 500 bootstrap replications to determine node reliabilities. The estimation of evolutionary divergence over sequence pairs between species clades were conducted using the Jukes-Cantor model.

SEM technique was performed in the Faculty of Sciences, University of Lisbon (FCUL), by Professor Graça Alexandre-Pires and Dr. Telmo Nunes. Both molecular biology procedures and genetic sequence analysis were performed in CIRAD under supervision and by Doctor Laëtitia Gardès and Doctor Claire Garros. The anatomical description of all body structures using COM technique was performed by Professor Jean-Claude Delécolle from University of Strasbourg.

2.3.4. Data assessment of *Culicoides* species found in Portugal (1952-2005)

Together with the above mentioned work, an exhaustive historical literature review on published studies about *Culicoides* species found in Portugal was performed (Cambournac, 1956, 1970a, b; Mellor *et al*., 1985; Capela *et al*., 1990, 1992, 1993, 1997, 2003; Lamblé *et al*., 1990; Pena, 2003; Diaz *et al*., 2005; Vila-Viçosa *et al*., 2009). Some specimens mentioned in this literature were revaluated using Mathieu *et al*., (2012) interactive identification key for female *Culicoides*.

2.3.5. Design of an identification key for Portuguese *Culicoides* fauna

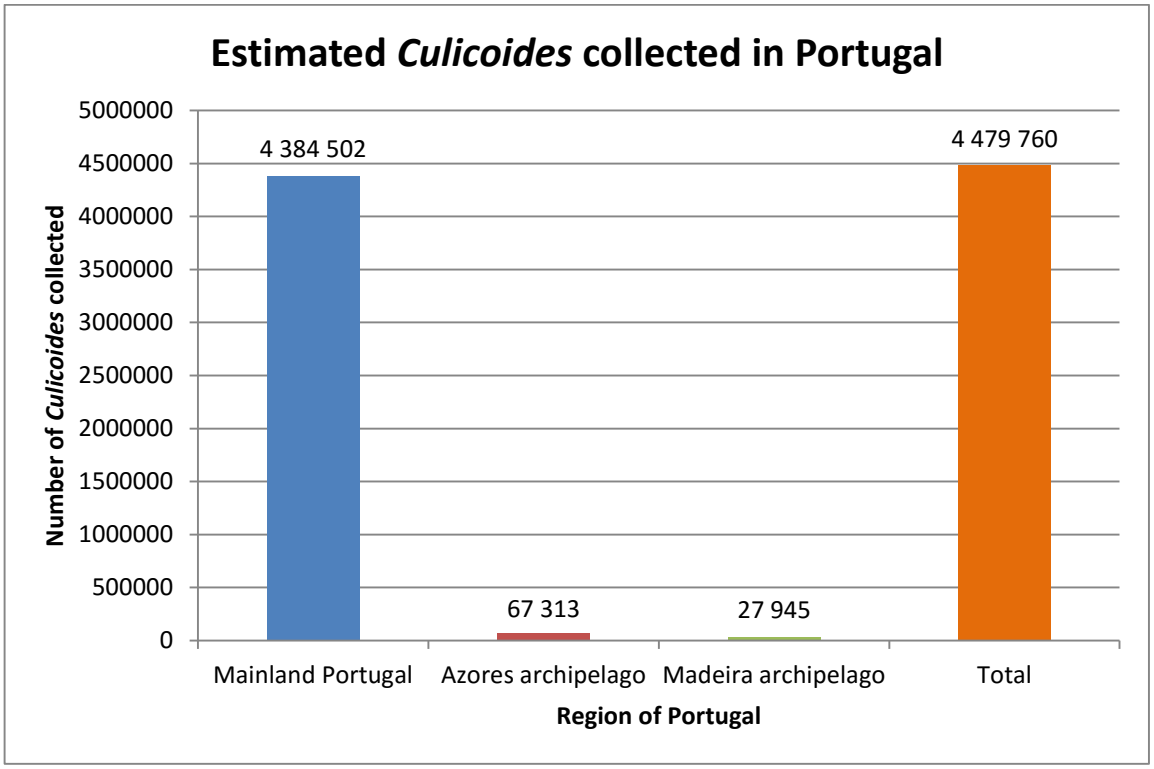
Data and photos obtained during this study were used to create an identification key for Portuguese *Culicoides* fauna.

2.4. Results

2.4.1. Distribution of *Culicoides* species in Portuguese territory

An estimated total of 4 479 760 *Culicoides* biting midges were collected in Portuguese territory during the NESP for BTD from 2005 to 2013 and VectorNet European network in 2015. From those, 4 384 502 *Culicoides* biting midges (97.87%) were collected in mainland Portugal, 67 313 (1.5%) in Azores and 27 945 (0.62%) in Madeira archipelagos, respectively (Figure 2.5.).

Figure 2.5. – Estimated *Culicoides* biting midges collected in Portugal during NESP (2005-2013) and VectorNet European network (2015).



The following species were identified during all period of the NESP exclusively by their wing pattern using SM and aimed by different identification keys (Delécolle, 1985; Rawlings, 1996; Pena, 2003; Mathieu, 2012):

1) *C. imicola*, *C. pulicaris* and midges belonging to Obsoletus group, since they were incriminated as Bluetongue vector species.

2) *C. punctatus*, *C. newsteadi*, *C. circumscriptus*, *C. maritimus* and *C. univittatus*, due to their characteristic wing pattern.

The other *Culicoides* species were labelled as *Culicoides* spp., since they were not priority for the NESP for BTD and were preserved in 96° alcohol.

2.4.1.1. Mainland Portugal

A total of 380.456 *Culicoides* specimens from species mentioned above were analysed, representing 8.68% of the total estimated for mainland Portugal. The relative frequencies of captured and analysed *Culicoides* biting midges are referred in Table 2.1.. The overall maximum capture of *C. imicola* and species from Obsoletus group between 2005 and 2010 per GU can be observed in Ribeiro *et al*. (2015).

Table 2.1. – Absolute and relative frequencies of estimated and analysed *Culicoides* collected during the NESP for BTD (2005-2013) and VectorNet European network (2015) in mainland Portugal.

***Culicoides* species**	**Total collected (estimated)**	**Total collected (% estimated)**	**Total analysed**	**Total analysed (%)**
C. imicola	3 109 345	70.92	180 832	47.52
Obsoletus group	216 349	4.93	71 216	18.72
C. pulicaris	3 575	0.08	1 811	0.48
C. punctatus	409 361	9.34	62 602	16.45
C. newsteadi	84 759	1.93	26 756	7.03
C. circumscriptus	14 535	0.33	3 213	0.84
C. maritimus	3 020	0.07	1 101	0.29
C. univittatus	5 696	0.13	3 627	0.95
Culicoides spp.	537 863	12.27	29 299	7.72
Total	4 384 502	100	380 456	100

From the 537 863 *Culicoides* biting midges labelled as *Culicoides* spp., 29 299 *Culicoides* (corresponding to 7.72% of the total *Culicoides* spp.) were morphologically evaluated and classified to species. The relative frequencies of these collected and analysed biting midges are represented in Table 2.2..

Table 2.2. – Absolute and relative frequencies of estimated and analysed *Culicoides* spp. collected during the NESP for BTD (2005-2013) and VectorNet European network (2015) in mainland Portugal.

***Culicoides* species**	**Total collected (estimated)**	**Total collected (% estimated)**	**Total analysed**	**Total analysed (%)**
C. achrayi	453 291	84.28	18 354	62.65
C. alazanicus[1]	1 647	0.31	36	0.12
C. albihalteratus	1 347	0.25	242	0.83
C. begueti	519	0.1	57	0.19
C. cataneii	37	0.007	19	0.06
C. clastrieri	20	0.004	14	0.05
C. corsicus	62	0.01	44	0.15
C. deltus[1]	496	0.09	183	0.62
C. derisor	127	0.02	47	0.16
C. fagineus	1	<0.001	1	0.003
C. fascipennis	6 192	1.15	915	3.12
C. festivipennis	4 926	0.92	1 256	4.29
C. gejgelensis	1 028	0.19	704	2.4
C. haranti	56	0.01	7	0.02
C. heliophilus[1]	145	0.03	15	0.05
C. heteroclitus	1 527	0.28	334	1.14
C. impunctatus	1 407	0.26	314	1.07
C. indistinctus	2 491	0.46	142	0.48
C. jumineri	123	0.02	57	0.19
C. jumineri near *C. bahrainensis*[1]	1 640	0.3	383	1.31
C. jurensis[1]	77	0.01	33	0.11
C. kibunensis/ C. atripennis	345	0.06	82	0.28
C. kingi[1]	1	<0.001	1	0.003
C. kurensis	1 551	0.29	249	0.85
C. longipennis	10 121	1.88	1 257	4.29
C. lupicaris[1]	168	0.03	44	0.15
C. malevillei[1]	3	<0.001	3	0.01
C. nubeculosus/ C. puncticollis/ C. riethi	1 041	0.19	303	1.03
C. odiatus	1 826	0.34	280	0.96
C. pallidicornis	1 044	0.19	225	0.77
C. paolae[1]	1	<0.001	1	<0.001
C. paradoxalis[2]	196	0.04	115	0.39
C. parroti/C. stigma/ C. helveticus	117	0.02	45	0.15
C. picturatus[1]	2 705	0.5	469	1.6
C. pseudopallidus	1 457	0.27	363	1.24
C. remmi[1]	50	0.009	5	0.02
C. riebi[1]	16	0.003	4	0.01
C. sahariensis	3 609	0.67	508	1.73
C. santonicus[1]	34 112	6.34	1 475	5.03
C. semimaculatus[1]	7	0.001	7	0.02
C. simulator[1]	3	<0.001	3	0.01
C. subfagineus[1]	1 379	0.26	301	1.03

Table 2.2. – Absolute and relative frequencies of estimated and analysed *Culicoides* spp. collected during the NESP for BTD (2005-2013) and VectorNet European network (2015) in mainland Portugal. (Continuation).

***Culicoides* species**	**Total collected (estimated)**	**Total collected (% estimated)**	**Total analysed**	**Total analysed (%)**
C. subfasciipennis	87	0.02	51	0.17
C. tbilisicus	387	0.07	70	0.24
C. vexans	480	0.09	282	0.96
Total	537 863	100	29 299	100

[1]Species reported for the first time in mainland Portugal. All species were caught during the NESP, with the exception of *C. paolae*, caught during VectorNet European network, July, 2015; [2]A new species for science.

As result, 16 *Culicoides* species (*C. alazanicus*, *C. deltus*, *C. heliophilus*, *C. jumineri* near *C. bahrainensis*, *C. jurensis*, *C. kingi*, *C. lupicaris*, *C. malevillei*, *C. paolae*, *C. picturatus*, *C. remmi*, *C. riebi*, *C. santonicus*, *C. semimaculatus*, *C. simulator* and *C. subfagineus*) were reported for the first time in mainland Portugal. The distribution of *Culicoides* species in mainland Portugal by GU is represented in Table 2.3. No *Culicoides* specimens were captured in the GUs 14 and 19. The synonyms of the abovementioned species are referred in Annex 2.3.

Table 2.3. – *Culicoides* species distribution in mainland Portugal by GUs.

***Culicoides* species**	**Mainland Portugal GUs**
C. achrayi	1, 4-7, 9-11, 13, 15, 17, 18, 22, 23, 25, 26, 28, 30, 31, 34, 36, 39, 40
C. alazanicus	10, 16, 18, 23, 40
C. albihalteratus	3, 4, 10, 13, 16-18, 22, 23, 25, 26, 30
C. begueti	4, 7, 10, 18, 24, 26, 28, 30, 31, 35-40, 44
C. cataneii	7, 20, 22, 23, 26, 30, 36, 39, 44
C. circumscriptus	3-5, 7, 10-13, 16-18, 20, 22-40, 42, 44
C. clastrieri	18, 22, 23, 36, 38, 40
C. corsicus	18, 23, 35, 36
C. deltus	4, 7, 9, 11, 18, 20, 25
C. derisor	4, 10, 18, 23, 26, 28, 30, 34, 36, 38, 40
C. fagineus	40
C. fascipennis	1, 4, 5, 7, 9, 12, 13, 16-18, 21, 25, 26, 28, 30, 31, 34, 36, 39, 40, 44
C. festivipennis	1, 4-7, 10-12, 15-18, 20-28, 30, 31, 34-36, 38, 40, 42, 44
C. gejgelensis	6, 7, 10, 12, 13, 16, 18, 20, 22-31, 36-38, 40, 42, 44
C. haranti	23, 24, 36, 38
C. heliophilus	4, 5, 7, 10, 15, 18
C. heteroclitus	1, 4, 7, 9-11, 17, 18, 20, 22, 23, 25, 26, 28, 30, 31, 35-40, 42
C. imicola	1, 5, 7-10, 13, 16-18, 20-40, 42, 44
C. impunctatus	4, 20, 30
C. indistinctus	4, 6, 7, 9-11, 15, 17, 18, 20, 22, 23, 25, 26, 30, 32, 35, 36, 39, 42
C. jumineri	5, 7, 17, 18, 23, 28, 30, 32, 35, 38, 39
C. jumineri near *C. bahrainensis*	4, 16, 18, 20, 22, 23, 25, 26, 28-31, 34-36, 38-40, 42, 44
C. jurensis	10, 18, 30, 31, 36, 38-40

Table 2.3. – *Culicoides* species distribution in mainland Portugal by GUs. (Continuation).

***Culicoides* species**	**Mainland Portugal GUs**
C. kibunensis/ C. atripennis[1]	1, 4, 16-18, 20, 23, 25, 26, 28, 30, 31, 35, 36, 38-40
C. kingi	11
C. kurensis	4, 16, 18, 22, 23, 25-27, 29-31, 34-36, 38
C. longipennis	7, 9-11, 18, 22, 23, 25-32, 34-36, 38-40, 42, 44
C. lupicaris	4, 20, 22, 25
C. malevillei	31, 36
C. maritimus	7, 12, 17, 23, 26, 27, 29-31, 34-36, 38-40, 42, 44
C. newsteadi	1, 3-7, 10-13, 15-18, 20-40, 42, 44
C. nubeculosus/ C. puncticollis/ C. riethi[2]	22-29, 32, 34-36, 38-40, 42, 44
C. odiatus	1, 4, 9, 10, 13, 18, 23, 25-28, 30, 31, 36, 38-40, 42
C. pallidicornis	1, 4, 8, 15-18, 26, 30, 34, 36, 40, 42
C. paolae	23
C. paradoxalis	4, 11, 18, 23, 24, 26, 28, 30, 31, 36, 38, 40, 42, 44
C. parroti/ C. stigma/ C. helveticus[3]	7, 11, 17, 18, 23, 24, 26, 28, 30, 31, 39, 40
C. picturatus	3-5, 7, 10, 11, 17, 18, 22-26, 36, 40
C. pseudopallidus	1, 4, 5, 7, 9-11, 18, 20, 23, 25, 26, 28-32, 34-36, 38-40
C. pulicaris	1, 3-12, 16-18, 20-26, 28-31, 36, 40, 42, 44
C. punctatus	1, 3-13, 15-18, 20-40, 42, 44
C. remmi	20
C. riebi	4, 26
C. sahariensis	28, 31, 36, 38-40, 42, 44
C. santonicus	5-7, 10-13, 17, 18, 22-26, 28-31, 35, 36, 38, 40
C. semimaculatus	28, 31, 36, 38, 40
C. simulator	7
C. subfagineus	4, 7, 11, 18, 22-24, 26, 28, 30, 31, 36, 38-40, 44
C. subfasciipennis	4, 7, 11, 15, 18, 20, 23, 25, 26, 30, 32, 34, 39, 42
C. tbilisicus	4, 10, 17, 26, 30, 36
C. univittatus	4, 5, 7, 10, 12, 13, 16-18, 20-31, 34-36, 38-40, 42, 44
C. vexans	4-7, 9, 11, 17, 18, 30
Obsoletus group	1, 3-13, 15-18, 20-40, 42, 44

[1]*C. atripennis* was only identified in GU 18; [2]*C. nubeculosus* specimens were only identified in GU 29. *C. puncticollis* species were identified in GUs 23, 25-29, 34, 36 and 38. No *C. riethi* specimens were identified; [3]Only *C. parroti* species was identified in the referred GUs.

From the 303 female specimens identified as *C. nubeculosus*/*C. puncticollis*/*C. riethi*, 90 (29.7%) were randomly selected and identified to species. All the 45 female specimens identified as *C. parroti*/*C. stigma*/*C. helveticus* were identified to species. Finally, from the 82 female specimens identified as *C. kibunensis*/*C. atripennis*, 21 (25.61%) were analysed to species, using the maximum length values of *C. kibunensis* wing, palpus and antenna as reference (Delécolle, 1985).

Only *C. nubeculosus* (in GU 29) and *C. puncticollis* (in GUs 23, 25-29, 34, 36 and 38) species were identified in the selected sample. Concerning *C. parroti*/*C. stigma*/*C. helveticus*, only *C. parroti* was identified in the referred GUs (Table 2.3.). Finally, both *C. atripennis* (in

GU 18) and *C. kibunensis* (in GUs 1, 4, 16-18, 20, 23, 25, 26, 28, 30, 31, 35, 36, 38-40) were identified.

Additionally, concerning *Culicoides* species referred in mainland Portugal, their seasonality is expressed in Table 2.4.

Table 2.4. – *Culicoides* species seasonality in mainland Portugal.

***Culicoides* species**	**Seasonality**	**Number of Months**
C. achrayi	April to October	7
C. alazanicus	April to November	8
C. albihalteratus	February to July, September	7
C. begueti	May to October	6
C. cataneii	January to March, June to July, September, November	7
C. circumscriptus	All year	12
C. clastrieri	March, May to October	7
C. corsicus	April to June, August to October	6
C. deltus	March to October	8
C. derisor	May to October	6
C. fagineus	October	1
C. fascipennis	All year	12
C. festivipennis	March to December	10
C. gejgelensis	All year	12
C. haranti	May, August, September	3
C. heliophilus	April to July	4
C. heteroclitus	April to November	8
C. imicola	All year	12
C. impunctatus	January to October, December	11
C. indistinctus	May to October	6
C. jumineri	April to November	8
C. jumineri near *C. bahrainensis*	February, April to November	9
C. jurensis	June to October	5
C. kibunensis/ *C. atripennis*	April to October	7
C. kingi	October	1
C. kurensis	April to November	8
C. longipennis	May to November	7
C. lupicaris	February to September	8
C. malevillei	July to September	3
C. maritimus	January, March, May to November	9
C. newsteadi	All year	12
C. nubeculosus/ *C. puncticollis*/ *C. riethi*	All year	12
C. odiatus	April to October	7
C. pallidicornis	May to October	6
C. paolae	July	1
C. paradoxalis	January, March, May to November	9
C. parroti/ *C. stigma*/ *C. helveticus*	January, April to August, October	7

Table 2.4. – *Culicoides* species seasonality in mainland Portugal (Continuation).

***Culicoides* species**	**Seasonality**	**Number of Months**
C. picturatus	April to August	5
C. pseudopallidus	April to November	8
C. pulicaris	All year	12
C. punctatus	All year	12
C. remmi	March, May, July and August	4
C. riebi	June to August	3
C. sahariensis	May to October	6
C. santonicus	March to June, August to November	9
C. semimaculatus	June to August	3
C. simulator	May	1
C. subfagineus	March to November	9
C. subfasciipennis	May to September, November	6
C. tbilisicus	May to October	6
C. univittatus	All year	12
C. vexans	April to June	3
Obsoletus group	All year	12

2.4.1.2. Azores archipelago

From the total of *Culicoides* biting midges collected in Azores archipelago during the NESP for BTD (2005-2012), 13 037 *Culicoides* (corresponding to 19.37% of the captured biting midges in this archipelago) were morphologically evaluated and classified to species or as belonging to Obsoletus group. Their distribution by each Azores archipelago island is referred in Table 2.5.

Table 2.5. – Absolute and relative frequencies of estimated and analysed *Culicoides* biting midges collected during the NESP for BTD (2005-2012) in each Azores archipelago island.

Azores archipelago island	***Culicoides* species**	**Total collected (estimated)**	**Total collected (% estimated)**[1]	**Total analysed**	**Total analysed (%)**[1]
Santa Maria	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group[2]	*Idem*	*Idem*	154 421 2 752	4.63 12.65 82.72
São Miguel	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group	5 031 8 11 335	30.73 0.05 69.23	63 8 1 351	4.43 0.56 95.01
Terceira	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group	*Idem*	*Idem*	22 38 135	11.28 19.49 69.23
Graciosa	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group[2]	*Idem*	*Idem*	11 1 204	5.09 0.46 94.44
São Jorge	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group	52 3 21 212	0.24 0.01 99.74	7 3 2 207	0.32 0.14 99.55
Pico	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group	730 3 23 798	2.98 0.01 97.01	580 3 4 364	11.72 0.06 88.22

Table 2.5. – Absolute and relative frequencies of estimated and analysed *Culicoides* biting midges collected during the NESP for BTD (2005-2012) in each Azores archipelago island. (Continuation).

Azores archipelago island	*Culicoides* species	Total collected (estimated)	Total collected (% estimated)[1]	Total analysed	Total analysed (%)[1]
Faial	*C. circumscriptus*[2] *C. newsteadi*[2] Obsoletus group	*Idem*	*Idem*	58 13 288	16.16 3.62 80.22
Flores	Obsoletus group[2]	1 028	100	338	100
Corvo	Obsoletus group[2]	*Idem*	*Idem*	16	100
	Total	67 313		13 037	

[1]Per island; [2]Species reported for the first time in the referred island; Idem: all *Culicoides* biting midges collected in the referred island were analysed and identified to species.

As result, midges belonging to Obsoletus group (*C. obsoletus* and *C. scoticus*) were signalized in all islands of Azores archipelago including those where they have never been reported before (Santa Maria, Graciosa, Flores and Corvo islands). Furthermore, *C. circumscriptus* and *C. newsteadi* were reported for the first time in 7 of the 9 islands of Azores archipelago: Santa Maria, São Miguel, Terceira, Graciosa, São Jorge, Pico and Faial.

2.4.1.3. Madeira archipelago

Finally, from the total of *Culicoides* biting midges collected in Madeira archipelago during the NESP for BTD (2005-2012), 6 114 *Culicoides* (corresponding to 21.88% of the total for Madeira archipelago) were evaluated and classified to species or as belonging to Obsoletus group. Their distribution in Madeira and Porto Santo islands is referred in Table 2.6.

Table 2.6. – Absolute and relative frequencies of estimated and analysed *Culicoides* biting midges collected during the NESP for BTD (2005-2012) in each Madeira archipelago island.

Madeira archipelago island	*Culicoides* species	Total collected (estimated)	Total collected (% estimated)[1]	Total analysed	Total analysed (%)[1]
Madeira	Obsoletus group	27 912	100	6 081	100
Porto Santo	*C. newsteadi* Obsoletus group	*Idem*	*Idem*	7 26	21.21 78.79
	Total	27 945		6 114	

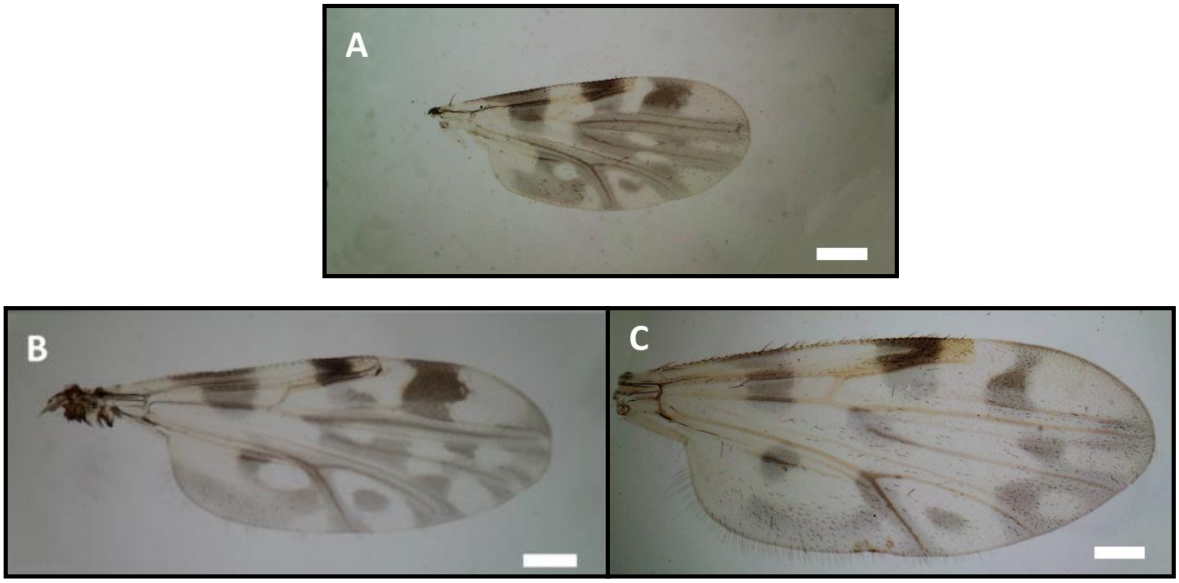
[1]Per island; Idem: all *Culicoides* biting midges collected in the referred island were analysed and identified to species.

2.4.2. *Culicoides paradoxalis* – a new species for science

The specimens labelled as "*C. sublupicaris*" were proved to be a new species for science, after an exhaustive work performed in collaboration with the FCUL, CIRAD (Montpellier) and the University of Strasbourg. The final name *Culicoides paradoxalis* Ramilo & Delécolle, 2013, was attributed to these specimens. The main morphological characteristics which

permit their differentiation from close related species, together with their characteristic wing pattern (Figure 2.6.), are described in Table 2.7 and represented in Figures 2.7., 2.8., 2.9. and 2.10..

Figure 2.6. – Wing pattern of *Culicoides paradoxalis* (A) and two close related species: *C. newsteadi* (B) and *C. lupicaris* (C) (Ramilo *et al*., 2013).



The presence of two isolated white spots in the middle of the m_1 and m_2 cells, a dark spot in the cua_1 cell and the absence of white spots in the terminal part of M_1, M_2 and CuA_1 veins are characteristic of the new species wing pattern. Scale bars: 200 µm.

Table 2.7. – Main morphological characteristics for *Culicoides paradoxalis* differentiation from close related species (Ramilo *et al*., 2013).

Anatomical structures	**Microscopic technique**	***C. lupicaris***	***C. newsteadi***	***C. paradoxalis***
Palpus 3rd segment	COM	14 sensilla basiconica individually disposed but absent in the segment anterior region	32 sensilla basiconica individually disposed into depressions along and around the segment	30-32 sensilla basiconica in only one side of the segment, sometimes grouped, and absent in the anterior region of the segment
	SEM	Sensilla basiconica in individual depressions	Sensilla basiconica in individual depressions	Sensilla basiconica in one single depression
Antennal 3rd flagellomere	SEM	Three individual sensilla coeloconica	Four individual sensilla coeloconica	Four individual sensilla coeloconica
Spine on the 4th tarsomere of middle legs	COM	Presence	Presence	Absence

Figure 2.7. – Sensilla basiconica of the 3rd palpus segment of *C. newsteadi* (A), *C. paradoxalis* (B) and *C. lupicaris* (C) (Ramilo *et al*., 2013).

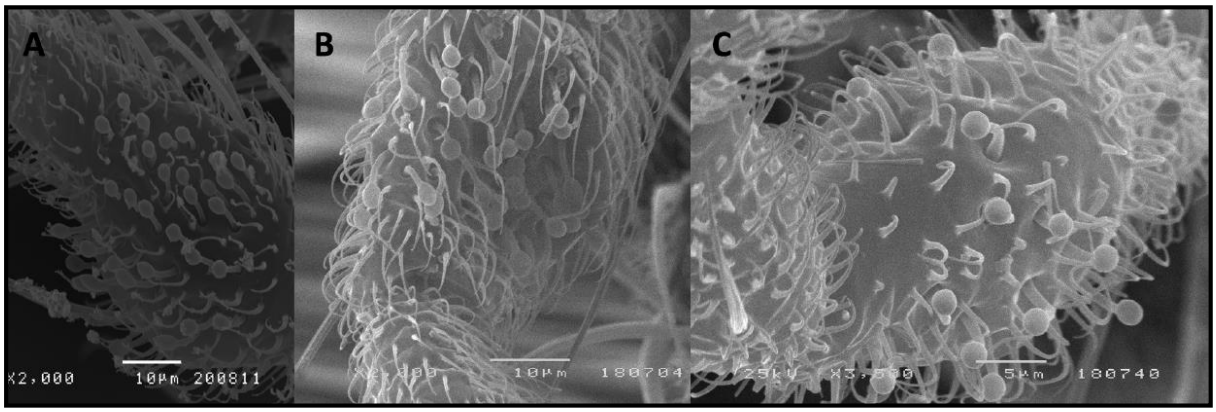
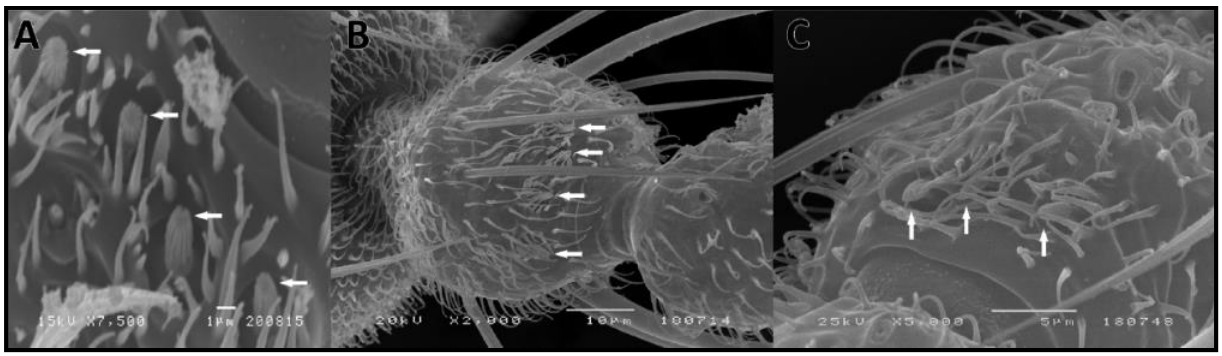
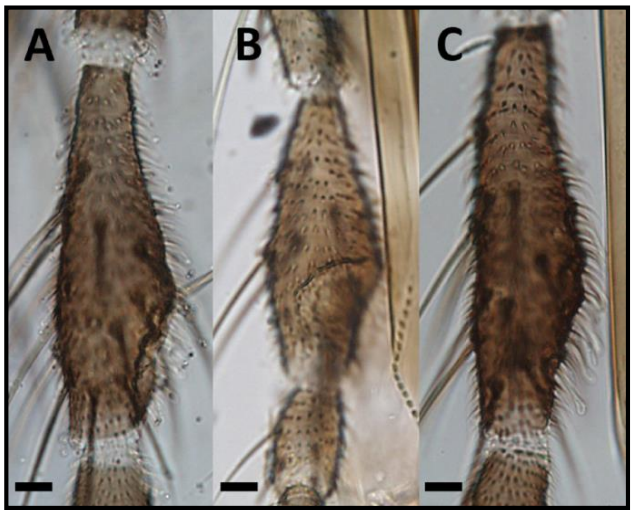


Figure 2.8. – Sensilla coeloconica on the 1st antennal flagellomere of *C. newsteadi* (A), *C. paradoxalis* (B) and *C. lupicaris* (C) (Ramilo *et al*., 2013).



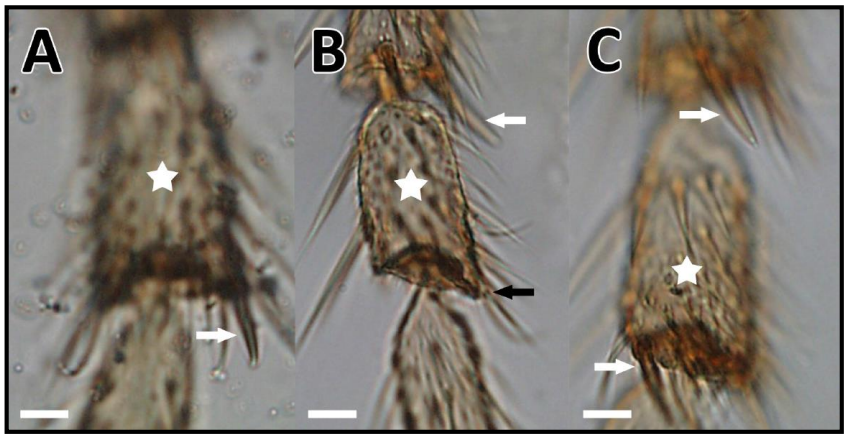
Sensilla coeloconica are marked by white arrows.

Figure 2.9. – 3rd palpus segment of *C. newsteadi* (A), *C. paradoxalis* (B) and *C. lupicaris* (C). (Ramilo *et al*., 2013).



Scale bars: 10 µm.

Figure 2.10. – 4th tarsomere of the middle legs of *C. newsteadi* (A), *C. paradoxalis* (B) and *C. lupicaris* (C) (Ramilo *et al*., 2013).



White arrow: presence of tarsal spine. Black arrow: absence of tarsal spine.
White star: 4th tarsomere of the middle legs. Scale bars: 10 μm

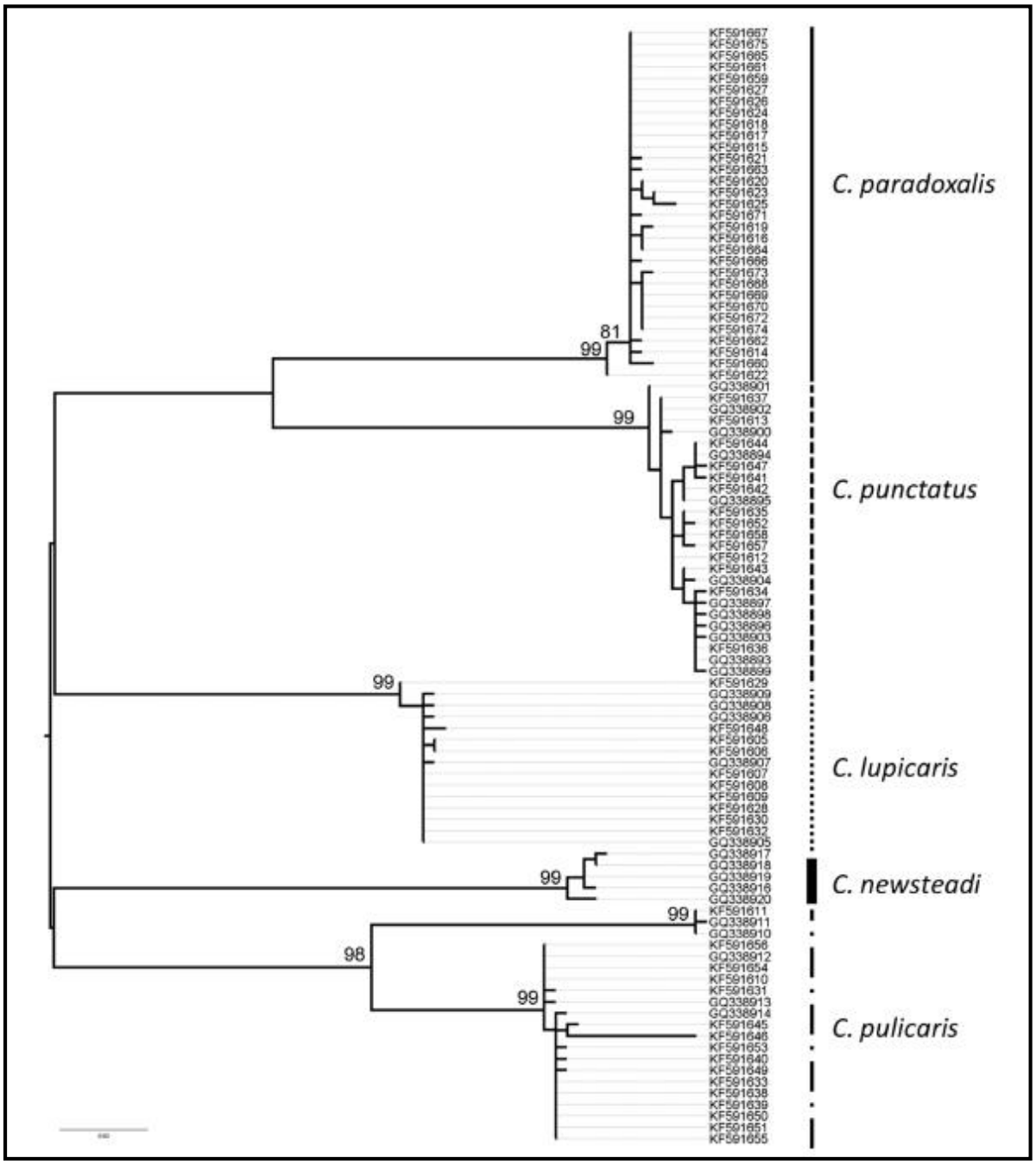
The mean genetic distance of the COI region between and within species is represented in Table 2.8., as well as the phylogenetic tree for *Culicoides* inferred from COI sequences (Figure 2.11.).

Table 2.8. – Mean genetic distance of the COI region between and within species (Ramilo *et al*., 2013).

Mean distance between species						**Mean distance within species**	
Species	1	2	3	4	5	Species	
1						*C. lupicaris*	0.0037
2	0.177					*C. pulicaris*	0.0352
3	0.207	0.206				*C. punctatus*	0.007
4	0.166	0.194	0.168			*C. paradoxalis*	0.0042
5	0.181	0.198	0.198	0.21		*C. newsteadi*	0.0079

1 – *Culicoides lupicaris*; 2 – *Culicoides pulicaris*; 3 – *Culicoides punctatus*; 4 – *Culicoides paradoxalis*; 5 – *Culicoides newsteadi*.

Figure 2.11. – Phylogenetic tree for *Culicoides* inferred from COI sequences (Ramilo *et al*., 2013).



The tree was unrooted. Bootstrap support values from maximum likelihood values are displayed on the nodes. Values lower than 70% are not shown.

2.4.3. Information obtained after data assessment of *Culicoides* species found in Portugal (1952-2005)

A comparison of *Culicoides* mentioned in the 1952-2005 and 2005-2013 periods and 2015 in Portuguese territory is referred in Tables 2.9. and 2.10.. Briefly, *C. brunnicans*, *C. chiopterus*, *C. furcillatus*, *C. pictipennis*, *C. riethi* and *C. shaklawensis* were not identified during the NESP for BTD in mainland Portugal, as well as *C. puncticollis* in Porto Santo island. *C. paolae* was identified for the first time during VectorNet European network, in July, 2015.

Table 2.9. – Comparison of *Culicoides* species referred in the 1952-2005 and 2005-2013 periods and 2015 in mainland Portugal

***Culicoides* species**	**1952-2005 Period**	**2005-2013 Period**	**2015**
C. achrayi	X	X	X
C. alazanicus		X	
C. albihalteratus	X	X	X
C. atripennis	X	X	
C. begueti	X	X	
C. brunnicans	X		
C. cataneii	X	X	
C. chiopterus	X		
C. circumscriptus	X	X	X
C. clastrieri	X	X	
C. corsicus	X	X	X
C. deltus		X	
C. derisor	X	X	X
C. fagineus	X	X	
C. fascipennis	X	X	X
C. festivipennis	X	X	X
C. furcillatus	X		
C. gejgelensis	X	X	X
C. haranti	X	X	X
C. heliophilus		X	
C. heteroclitus	X	X	X
C. imicola	X	X	X
C. impunctatus	X	X	
C. indistinctus	X	X	X
C. jumineri	X	X	
C. jumineri near *C. bahrainensis*		X	X
C. jurensis		X	
C. kibunensis	X	X	X
C. kingi		X	
C. kurensis	X	X	X
C. longipennis	X	X	X
C. lupicaris		X	X
C. malevillei		X	
C. maritimus	X	X	
C. newsteadi	X	X	X
C. nubeculosus	X	X	
C. nuntius	X		
C. obsoletus	X	X	X
C. odiatus	X	X	X
C. pallidicornis	X	X	X
C. paolae			X
C. paradoxalis		X	
C. parroti	X	X	X
C. pictipennis	X		
C. picturatus		X	
C. pseudopallidus	X	X	X
C. pulicaris	X	X	X
C. punctatus	X	X	X
C. puncticollis	X	X	X
C. remmi		X	

Table 2.9. – Comparison of *Culicoides* species referred in the 1952-2005 and 2005-2013 periods and 2015 in mainland Portugal (Continuation).

***Culicoides* species**	**1952-2005 Period**	**2005-2013 Period**	**2015**
C. riebi		X	
C. riethi	X		
C. rochenus	X		
C. sahariensis	X	X	
C. santonicus		X	X
C. scoticus	X	X	X
C. semimaculatus		X	
C. shaklawensis	X		
C. simulator		X	
C. subfagineus		X	
C. subfasciipennis	X	X	X
C. tbilisicus	X	X	X
C. univittatus	X	X	X
C. vexans	X	X	

Table 2.10. - Comparison of *Culicoides* species referred in the 1952-2005 and 2005-2013 periods in Azores and Madeira archipelagos.

***Culicoides* species**	**1952-2005 Period**	**2005-2013 Period**	**Portugal region**
C. obsoletus	X	X (as Obsoletus group)	Madeira archipelago
C. scoticus	X		
C. newsteadi	X	X	Porto Santo island
C. puncticollis	X		
C. obsoletus	X[1]	X[2]	Azores archipelago
C. scoticus			
C. circumscriptus		X[3]	
C. newsteadi			

[1]In São Miguel, Terceira, São Jorge, Pico and Faial islands; [2]In all islands of Azores archipelago; [3]In Santa Maria, São Miguel, Terceira, Graciosa, São Jorge, Pico and Faial islands.

Some specimens observed by Pena (2003) and Vila-Viçosa *et al*., (2009) have morphological characteristics similar to other *Culicoides* species (Table 2.11).

Table 2.11. – *Culicoides* species observed by Pena (2003) and Vila-Viçosa *et al*., (2009) and similar species.

***Culicoides* species**	**Identified by**	**Similar species[1]**
C. duddingstoni *C. impunctatus*[2] *C. heliophilus*[3] *C. jurensis* *C. marcleti* *C. obsoletus* *C. pseudopallidus*	Vila-Viçosa *et al*., 2009	*C. festivipennis* *C. fagineus* and *C. subfagineus* *Culicoides* spp. *C. indistinctus* *C. corsicus* *C. montanus*[4] *C. jumineri* near *C. bahrainensis*[5]
C. brunnicans *C. jumineri*	Pena, 2003 and Vila-Viçosa *et al*., 2009	*C. santonicus* *C. jumineri* near *C. bahrainensis*[5]

Table 2.11. – *Culicoides* species observed by Pena (2003) and Vila-Viçosa *et al*., (2009) and similar species (Continuation).

***Culicoides* species**	**Identified by**	**Similar species**
C. clintoni *C. truncorum*	Pena, 2003	*C. albihalteratus* *C. gejgelensis*

[1]According to Mathieu *et al*., 2012; [2]Name attributed to two different specimens; [3]Although the specimen is not *C. heliophilus*, available photographs do not allow species identification; [4]Male specimen (Mathieu, 2011). [5]Specimens similar to *C. bahrainensis* according to Delécolle, personal communication, March 22, 2010 and Mathieu *et al*., 2012;

2.4.4. *Culicoides* species captured in cattle and horse farms

Culicoides species captured in the framework of VectorNet European network (2015) near cattle and horse farms, aiming to determine if *Culicoides* fauna was different in each situation, are described in Table 2.12.

Table 2.12. – *Culicoides* species captured near cattle and horse farms during VectorNet field work (2015).

***Culicoides* species**	**Cattle**	**Horses**
C. achrayi	X	X
C. albihalteratus	X	X
C. circumscriptus	X	X
C. corsicus		X
C. derisor	X	
C. fascipennis		X
C. festivipennis	X	X
C. gejgelensis	X	
C. haranti		X
C. heteroclitus	X	X
C. imicola	X	X
C. indistinctus	X	X
C. jumineri near *C. bahrainensis*	X	X
C. kibunensis	X	X
C. kurensis	X	X
C. longipennis	X	X
C. lupicaris	X	
C. newsteadi	X	X
C. obsoletus	X	X
C. odiatus		X
C. pallidicornis	X	X
C. paolae	X	
C. parroti	X	X
C. pseudopallidus	X	X
C. pulicaris	X	X
C. punctatus	X	X
C. puncticollis	X	
C. santonicus		X
C. scoticus	X	X
C. subfasciipennis	X	X
C. tbilisicus		X
C. univittatus	X	X

2.4.5. Identification key for *Culicoides* female specimens

An easy identification key for *Culicoides* female specimens [concerning the Portuguese *Culicoides* fauna found during the NESP (2005-2013) and VectorNet European network (2015)] was elaborated, using different photographs from several morphological structures of these biting midges (Figure 2.12.).

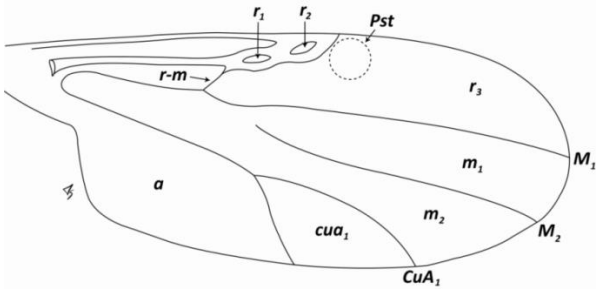
Species were divided into three major groups:

1) Those with a characteristic wing pattern and do not require insect mounting.

2) Those with similar wing pattern but do not require insect mounting.

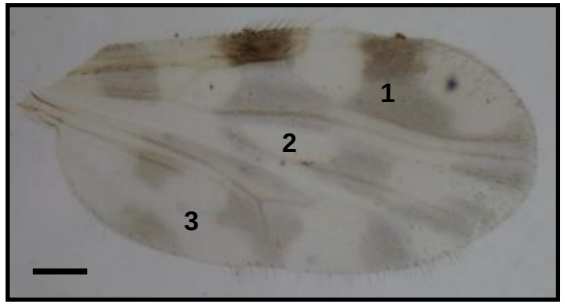
3) Those with indistinguishable wing pattern and do require insect mounting for identification by analysis of other morphological structures.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted).



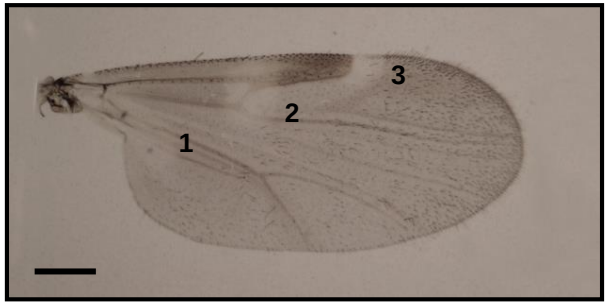
Cells and veins of a *Culicoides* biting midge wing: r-m – Radio-medial crossvein; r_1 – First radial cell; r_2 – Second radial cell; r_3 – Third radial cell; m_1 – First medial cell; m_2 – Second medial cell; cua_1 – Anterior cubital vein; a – Anal cell; Pst – Poststigmatic pale spot; M_1 – First medial vein; M_2 – Second medial vein; CuA_1 – First branch of anterior cubital vein (reproduced with kindly permission of M.Sc. Marcos Santos).

A – Species with characteristic wing pattern (do not require insect mounting):



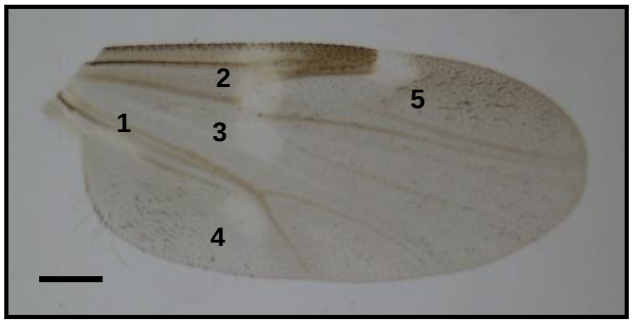
Culicoides imicola – The presence of a characteristic dark spot in the r_3 cell (1), a white almond shaped pattern in the proximal part of m_1 cell (2) and a white vertical hourglass shaped pattern in the anal cell (3) is typical of this species. Scale bar: 100 µm.

Culicoides circumscriptus – The presence of a dark circle in the r-m crossvein (1) is typical of this species. Scale bar: 200 µm.

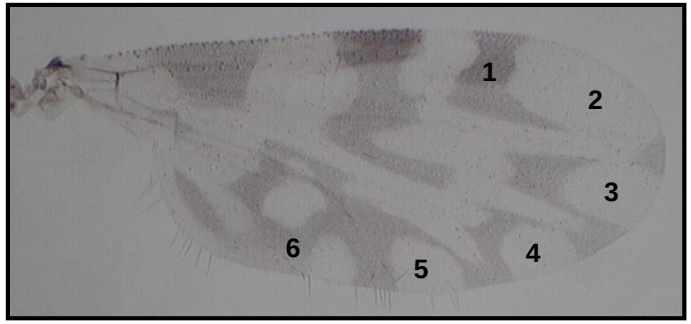


Culicoides achrayi – This species has a pronounced dark wing pattern with three characteristic white spots: in the wing proximal region (1), in the r-m crossvein (2) and a poststigmatic pale spot (3). Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).



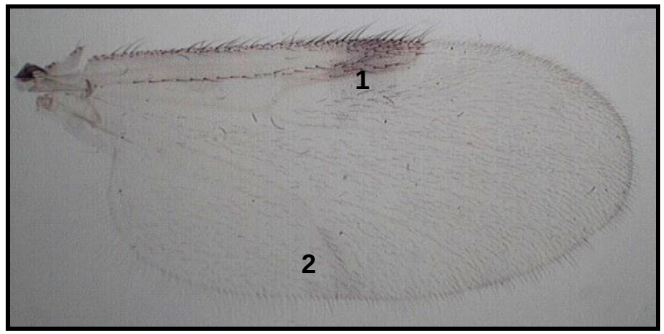
Culicoides fascipennis – This species has a pronounced dark wing pattern with a very small white spot in the wing posterior region (1) and four additional white spots: in the r-m crossvein (2), in the m cell (3), in the anal cell (4) and a poststigmatic pale spot (5). Sometimes, the white spot in m cell can be fused with that of the r-m crossvein. Scale bar: 200 µm.



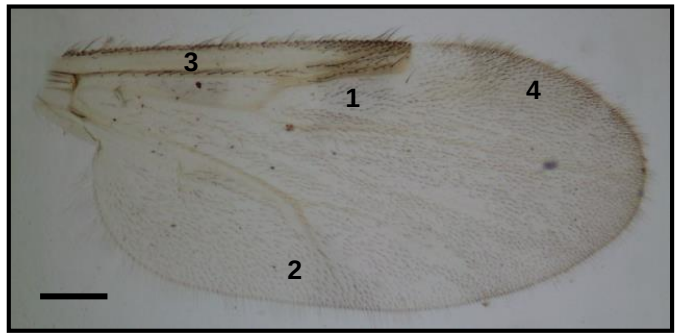
Culicoides shaklawensis – This species has a characteristic wing pattern with a dark spot in the r_3 cell (1), white spots at the apex of the r_3, m_1, m_2 and cua_1 cells (2, 3, 4 and 5, respectively) and two isolated white spots in the anal cell (6) (Mathieu *et al*., 2012).

B – Species with similar wing pattern (do not require insect mounting):

1. Light wing pattern with a dark spot covering the two radial cells (1) and another in the left branch of the CuA vein (2):



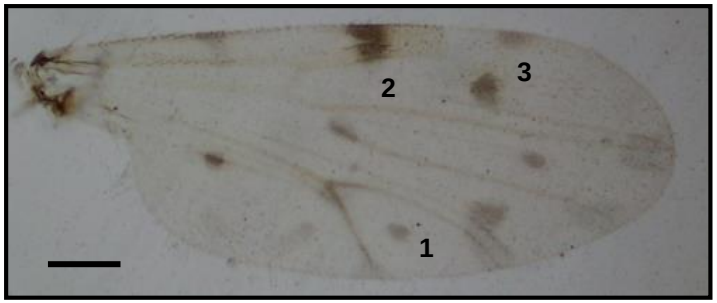
Culicoides brunnicans (Mathieu *et al*., 2012)



Culicoides santonicus – Dark spots in the r-m crossvein proximal region (3) and in the anterior part of the r_3 cell (4). Scale bar: 200 µm.

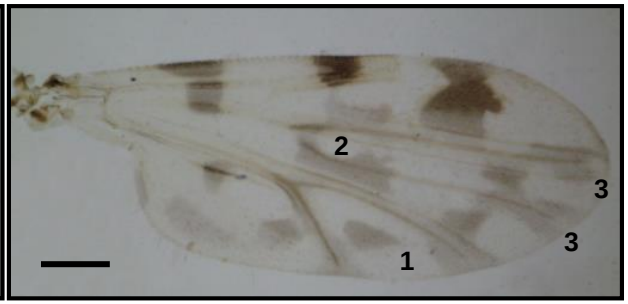
Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

2. Wings with a dark spot in the cua_1 cell (1):

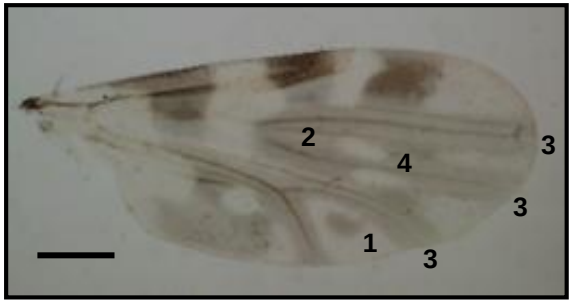


Culicoides pulicaris – This wing pattern lacks a dark spot below the r_2 cell (2). The dark spots at the r_3 cell (3) can be united like in *Culicoides punctatus*. Scale bar: 200 µm.

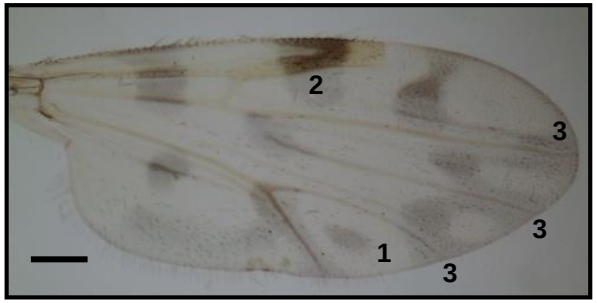
Culicoides punctatus – This wing pattern has a dark spot below the r_2 cell (2), unlike *Culicoides pulicaris*. Scale bar: 200 µm.



Culicoides newsteadi – This wing pattern has a dark spot in the proximal part of the M_2 vein (bigger than that of *Culicoides punctatus*), surrounding a white spot in the proximal part of m_1 cell (2). There is always, at least, one white spot at the apex of the M_1, M_2 and CuA_1 veins (3). Scale bar: 200 µm.



Culicoides paradoxalis – The white spot above the proximal part of M_2 vein is not present like in *Culicoides newsteadi* (2). There are no white spots in the apex of the M_1, M_2 and CuA_1 veins (3). The white spots in the middle of the m_1 and m_2 cells are not fused (4). The dark spot in cua_1 cell may be linked with the black spot of the CuA_1 vein (1). Scale bar: 200 µm.

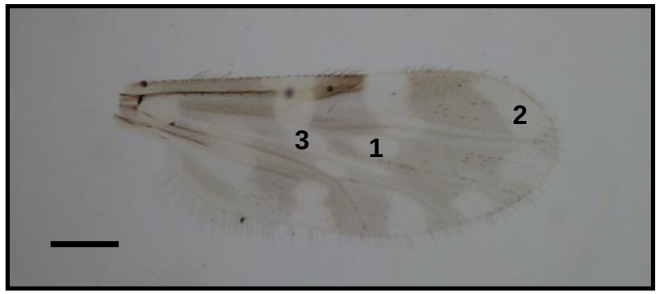


Culicoides lupicaris – A continuous dark spot begins in the wing anterior margin and goes through the two radial cells (2). White spots in the apex of the M_1, M_2 and CuA_1 veins can be absent (3). Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

3. Wings with one isolated white spot in the proximal/middle part of the m_1 cell (1):

Culicoides festivipennis – A white spot is present in the middle part of the M_1 vein, below the poststigmatic pale spot (2). The white spot in the m cell is not fused with any white spot around it and does not extend itself into the m_2 cell (3). Sometimes, two white spots can be observed in the basal part of m_2 cell (4). Scale bar: 200µm.



Culicoides clastrieri – The r_3 white spot is bigger than that observed in *Culicoides festivipennis* (2). The m cell white spot is connected with the r-m crossvein white spot and expands itself into the m_2 cell (3). Scale bar: 200µm.

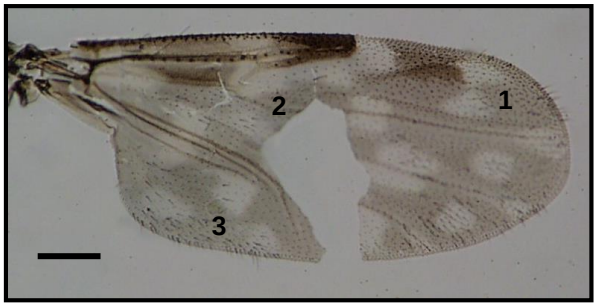
Culicoides longipennis – There is no white spot in the middle of the M_1 or M_2 veins (2). Scale bar: 200µm.

Culicoides sahariensis – The white spot in the middle of the m_1 cell reaches the M_1 and M_2 veins (1). The post-stigmatic spot does not reach the M_1 vein (2). Scale bar: 200µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

4. Wings with two isolated white spots in the r_3 cell (1):

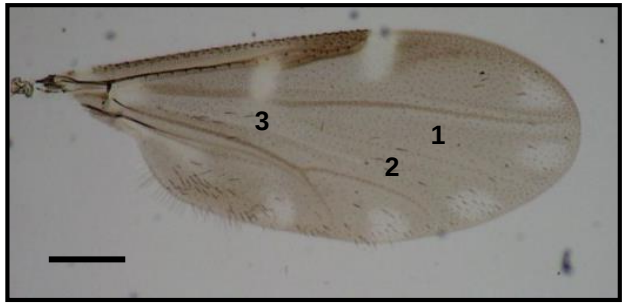
Culicoides kingi – The presence of two isolated white spots in cua_1 cell, surrounded by a dark area, is typical of this species (2). m cell white spot fused with the r-m crossvein white spot (3). Two fused white spots in anal cell (4). Scale bar: 100 µm.



Culicoides paolae – Wing pattern similar to *Culicoides kingi* but with two fused white spots in cua_1 cell, surrounded by a black area (not visible); m cell white spot not fused with the r-m crossvein white spot (2). Anal cell with two isolated white spots (3). (Incomplete wing obtained from a single observed specimen). Scale bar: 200 µm.

5. Wings with absence of white spots in the middle of the m_1 (1) and m_2 (2) cells:

Culicoides alazanicus – Presence of an isolated m cell white spot (3). Pronounced black lines between M_1, M_2 and CuA_1 veins (4). Scale bar: 200 µm.

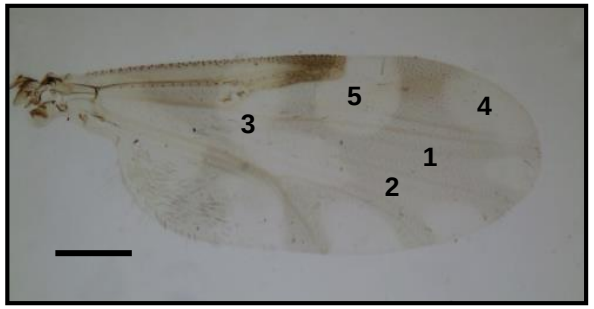


Culicoides haranti – Absence of the m cell white spot (3). Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

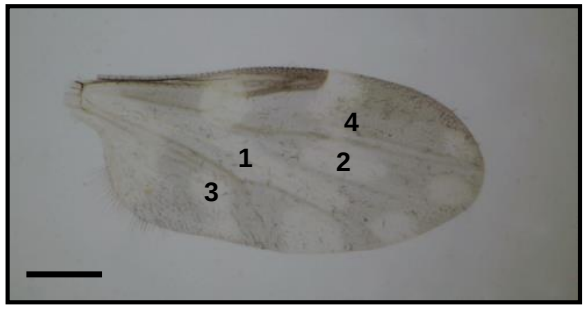
6. Wings with absence of white spots in the middle of the m_1 (1) and m_2 (2) cells and m cell white spot fused with r-m crossvein white spot (3):

Culicoides picturatus – White spots in the distal part of the r_3 and m_1 cells are bad defined (4). Scale bar: 200 µm.



Culicoides simulator – Presence of a white spot in the distal part of r_3 cell, bigger than the distal m_1 cell white spot (4). Poststigmatic pale spot starts in the wing margin and crosses the M_1 vein (5). m cell white spot expands slightly into the m_1 and m_2 cells (3). Scale bar: 200 µm.

7. Wings where the m cell white spot expands itself into the m_2 cell (1) and presence of a white spot in the middle of m_1 cell (2):



Culicoides maritimus – Presence of two white spots in anal cell (fused or separated) (3). Presence of a small white spot in the middle of the M_1 vein (4). Scale bar: 200 µm.

Culicoides univittatus – White spots of the distal part of the r_3 and m_1 cells close before they reach wing margin (3). When this feature is difficult to observe, it can be confounded with *C. gejgelensis*, *C. cataneii* and *C. pictipennis*. Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

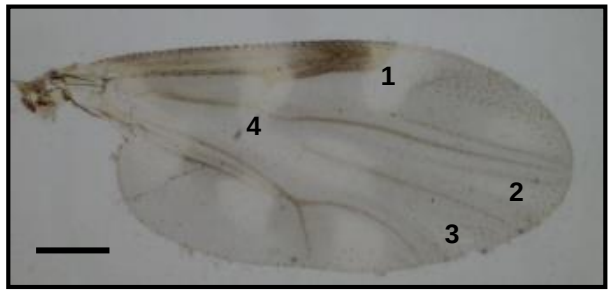
8. Wings without pale spots in the distal part of r_3 and m_1 cells (1):

Culicoides jumineri near *Culicoides bahrainensis* – This wing does not possess the white spot in the distal part of m_2 cell like in *Culicoides jumineri* (2). The white spot in the m cell extends itself into the m_2 cell (3). Scale bar: 200 µm.

Culicoides kurensis – Although not easy to observe, a smooth grey patch isolates the white spot in the m_2 cell from the white spot of the r-m crossvein (2). Scale bar: 200 µm.

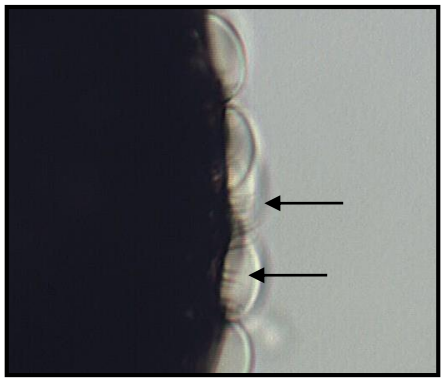
C – Undistinguishable wing patterns (requires insect mounting):

1. Wings with a characteristic poststigmatic pale spot (1) and presence of two isolated white spots which can cross the entire m_1 (2) and m_2 (3) cells, being this last one fused with the m cell white spot (4):

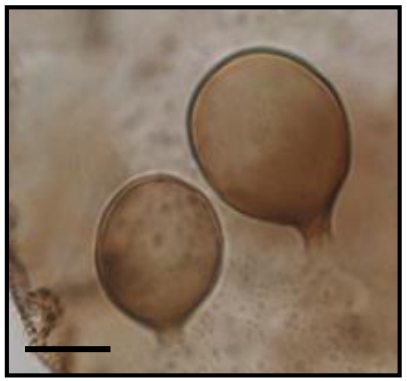


Includes species from Obsoletus group: *Culicoides chiopterus*, *Culicoides dewulfi, Culicoides montanus, Culicoides obsoletus* (left image) and *Culicoides scoticus*. Scale bar: 200 µm.

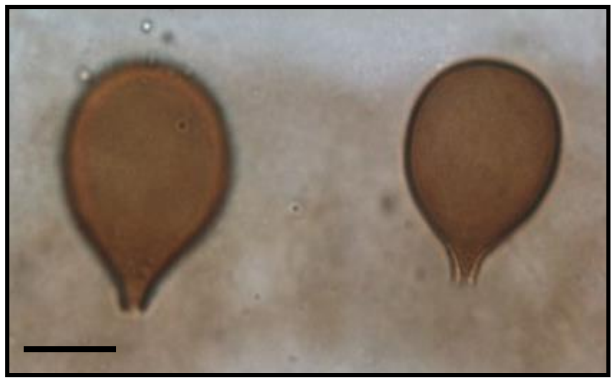
Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).



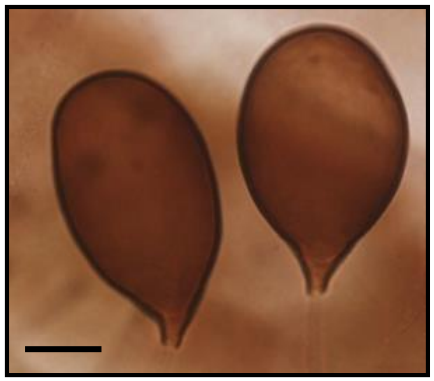
Culicoides chiopterus – This species has scattered and short interfacetal hairs (black arrows) (Mathieu *et al*., 2012).



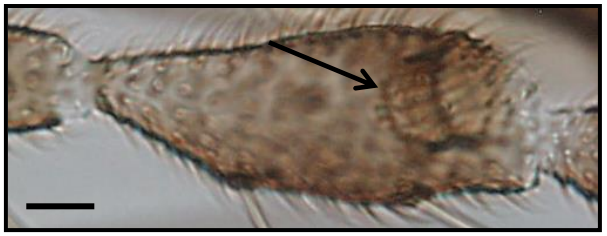
Culicoides dewulfi – This species has a two different sized spermathecae. Scale bar: 20 µm.



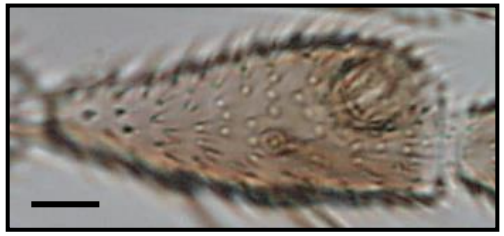
Culicoides obsoletus and *C. montanus* – Both species have an equal sized spermathecae with ≤ 62.5 µm. Scale bar: 20 µm.



Culicoides scoticus – This species has equal sized spermathecae with ≥ 57 µm. Scale bar: 20 µm.



Culicoides montanus – This species has a deeper sensorial pit (black arrow) when compared with *C. obsoletus* and *C. scoticus*. Scale bar: 10 µm.

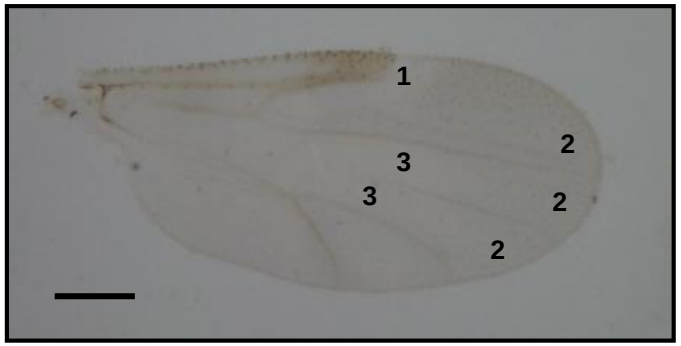


Culicoides obsoletus – The 3rd palpus segment of this species has a ratio length/width ≤ 2.7. Scale bar: 10 µm.

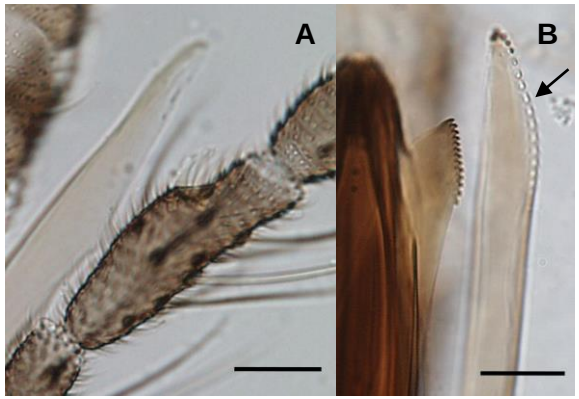
Culicoides scoticus – The 3rd palpus segment of this species has a ratio length/width ≥ 2.7. Scale bar: 10 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

Note: *Culicoides albihalteratus* wing pattern, although different, can be very similar to that of Obsoletus group species and is easily confounded as belonging to this group. For this reason it is included here.



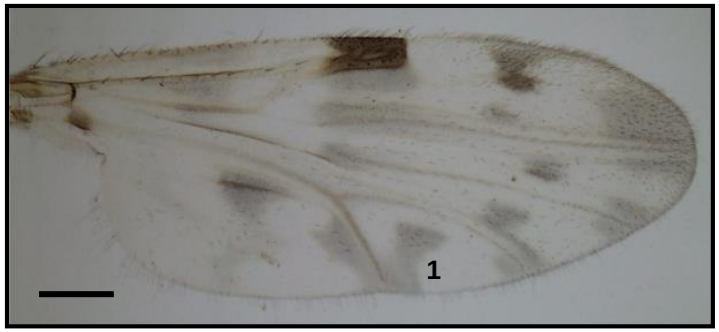
Culicoides albihalteratus – the poststigmatic pale spot has a different shape from that observed in species from Obsoletus group (1). There are no white spots in the distal part of the r_3, m_1 and m_2 cells (2) and the white spots are very difficult to observe (3) (aspect that can also occur in Obsoletus group species). Scale bar: 200 µm.



Culicoides albihalteratus (A) does not have teeth in the mandible while species from Obsoletus group have. B – *Culicoides scoticus*. Black arrow – teeth. Scale bars: 20 µm

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

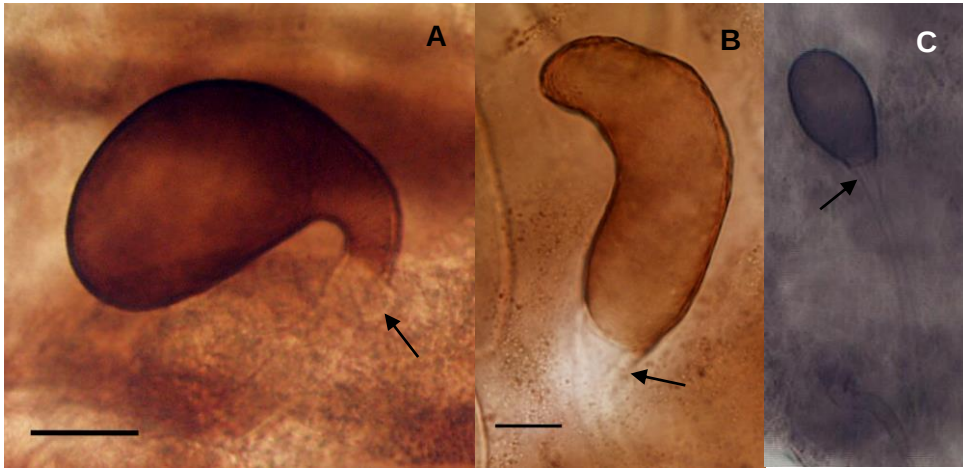
2. Wings with a dark spot in the cua_1 cell connected with the left branch of the CuA vein (1):



Includes: *Culicoides nubeculosus* (left image), *Culicoides puncticollis* and *C. riethi*. Scale bar: 200 µm.

Culicoides nubeculosus (A) – Almost all spermatheca is curved on itself. Presence of an enlarged ring in the beginning of the spermathecal duct (black arrow). Spermathecal duct longer than spermatheca. Scale bar: 20 µm.

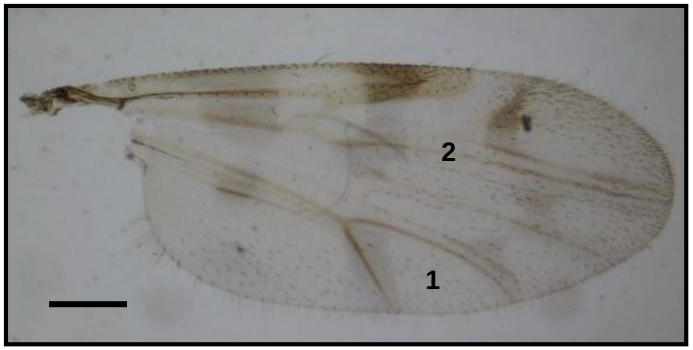
Culicoides puncticollis (B) – Spermatheca can be straight or its anterior portion slightly curved. Absence of an enlarged ring in the beginning of the spermathecal duct (black arrow). Spermathecal duct in the length of spermatheca. Scale bar: 20 µm.



Culicoides riethi (C) - Spermatheca can be straight or its anterior portion slightly curved. Absence of an enlarged ring in the beginning of the spermathecal duct (black arrow). Spermathecal duct longer than spermatheca (Mathieu *et al*., 2012).

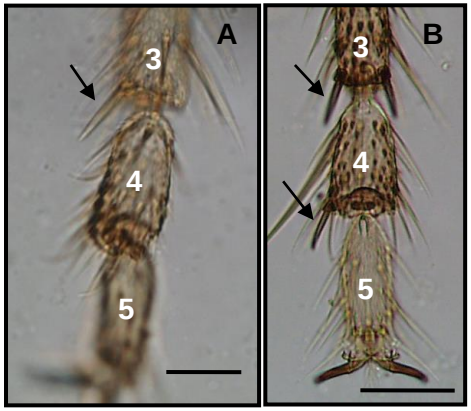
Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

3. Wing pattern similar to *Culicoides lupicaris* but without a dark circle in the cua_1 cell (1):

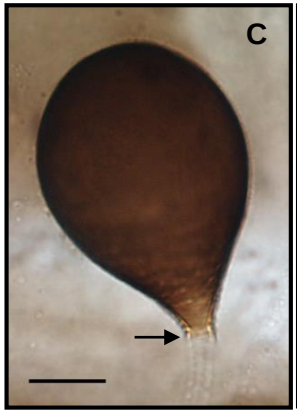


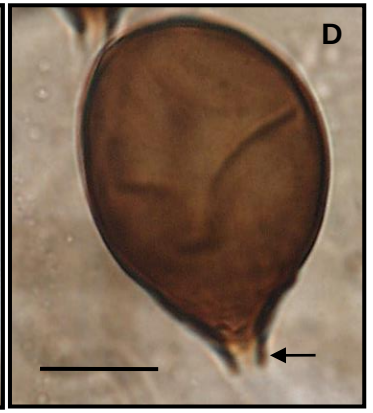
Absence of a dark spot in the middle of the M_1 vein (2). Includes: *Culicoides deltus* (upper image), *Culicoides impunctatus* and *Culicoides remmi*. Scale bar: 200 µm

Presence of a dark spot in the middle of the M_1 vein (2). Includes: *Culicoides fagineus* and *Culicoides subfagineus* (upper image). Scale bar: 200 µm



Culicoides impunctatus (A) has spines from the 1st to the 3rd tarsomere of middle legs, while *Culicoides deltus* (B) and *Culicoides remmi* have spines from the 1st to the 4th tarsomere of middle legs. Black arrows – tarsomere spines; 3 to 5 – 3rd to 5th tarsomere. Scale bars: 20 µm.





Culicoides deltus (C) has a shorter spermathecae pigmented neck than *Culicoides remmi* (D). Black arrow: spermathaecae pigmented neck. Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

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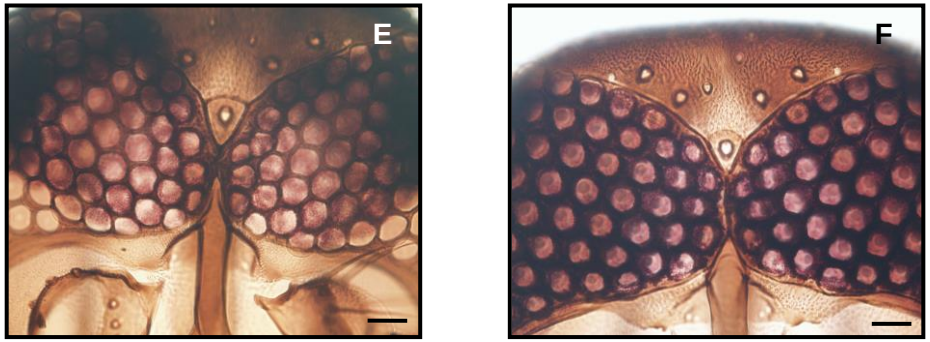
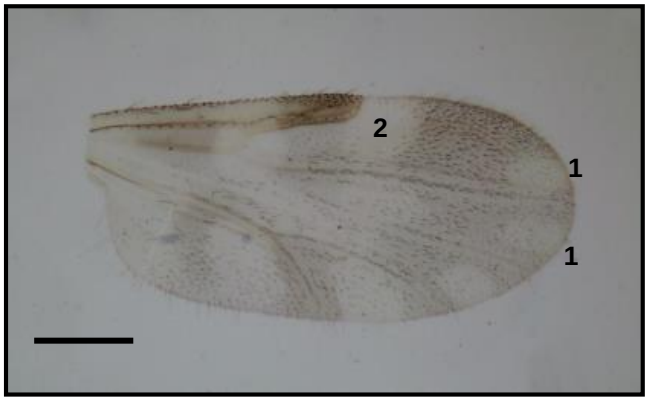


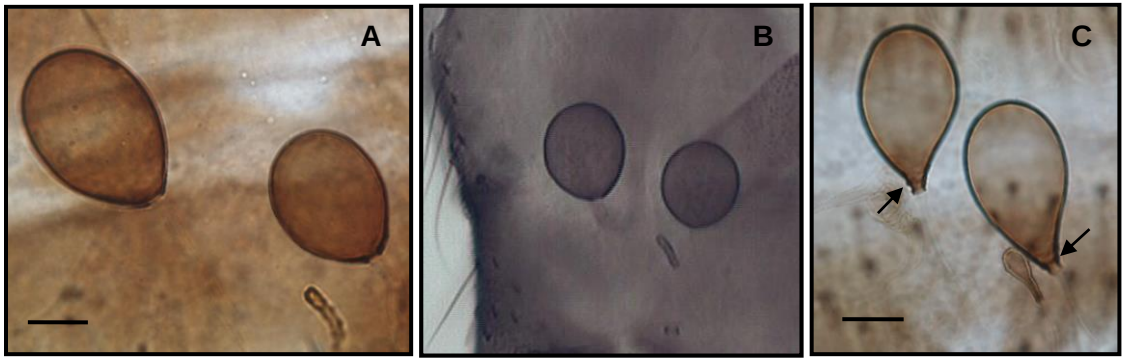
The eyes are joined for a shorter distance in *Culicoides subfagineus* (E) than in *Culicoides fagineus* (F). Scale bars: 20 µm.

4. Wing pattern similar to *Culicoides univittatus* but the white spots in the distal part of the r_3 and m_1 cells do not close before wing margin (1):

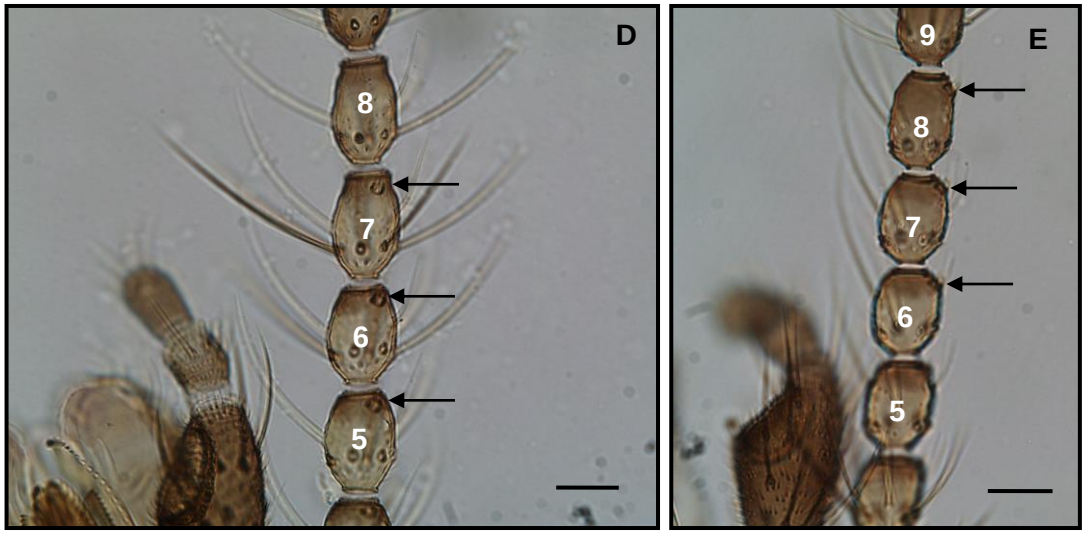


Includes: *Culicoides cataneii*, *Culicoides gejgelensis* (left image), *Culicoides pictipennis* and *Culicoides univitattus* (when its wing pattern characteristics are difficult to observe). In *Culicoides pictipennis*, the poststigmatic pale spot (2) covers more than 1/3 of the r_2 cell. Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).



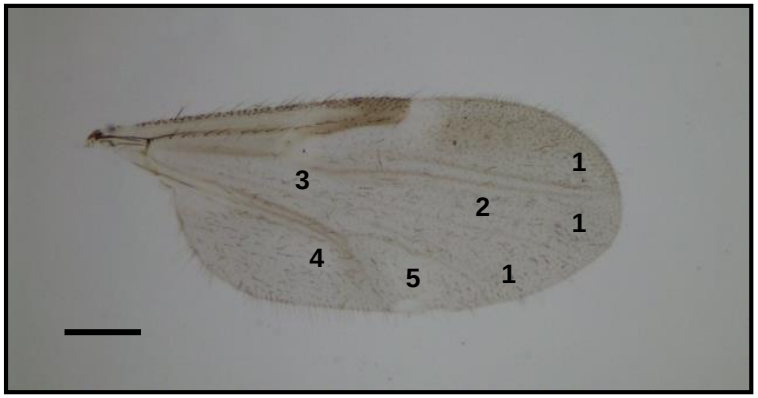
Culicoides univittatus (A) has unequal spermathecae length, while *Culicoides pictipennis* (B), *Culicoides cataneii* and *Culicoides gejgelensis* (C) have equal spermathecae length. This last species also have a small sclerotic ring in their spermathecae pigmented neck (black arrows). *Culicoides pictipennis* and *Culicoides univittatus* do not possess a pigmented neck, while *Culicoides cataneii* and *Culicoides gejgelensis* have a pigmented neck. Scale bars: 20 µm. Image B: Mathieu *et al*., 2012.



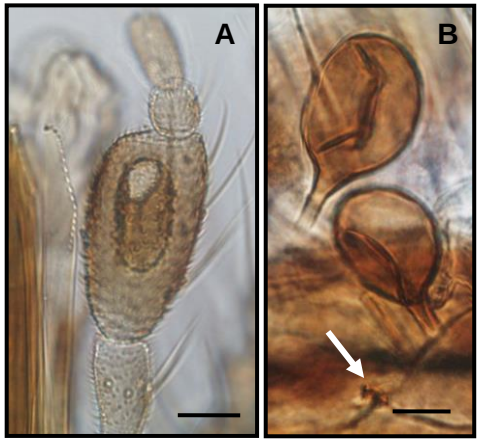
Culicoides cataneii (D) has sensilla coeloconica from the 5th to the 7th antennal flagellomere and *Culicoides gejgelensis* (E) from the 5th to the 8th antennal flagellomere. Black arrows – sensilla coeloconica; 5 to 9 – 5th to 9th antennal flagellomere. Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

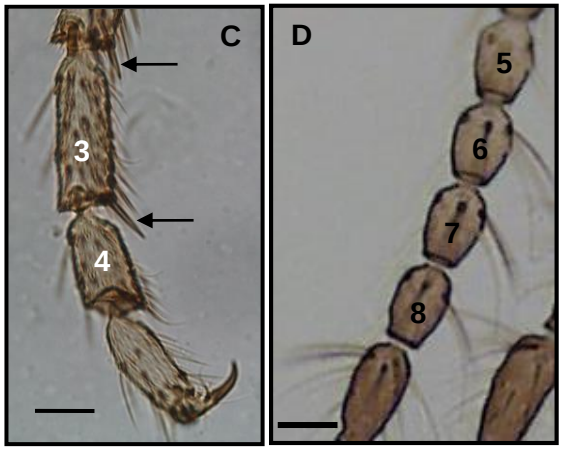
5. Dark wings with no white spots in the distal part of the r_3, m_1 and m_2 cells (1) and no white spot in the middle of m_1 cell (2):



Includes: *Culicoides heteroclitus* (left image), *Culicoides pseudopallidus*, *Culicoides semimaculatus* and *Culicoides subfasciipennis*. These species have a high level of wing pattern intraspecific variation. Concerning the m cell white spot (3), in *C. semimaculatus* it can be absent, present or present and fused with the r-m crossvein white spot. The anal cell white spot (4) can be absent in *C. heteroclitus*, *C. pallidicornis* and *C. subfasciipennis*. Finally, cua_1 white spot (5) can be absent in *C. heteroclitus* and *C. pallidicornis*. *C. heteroclitus* and *C. pseudopallidus* species are very difficult to distinguish between them. Scale bar: 200 µm.

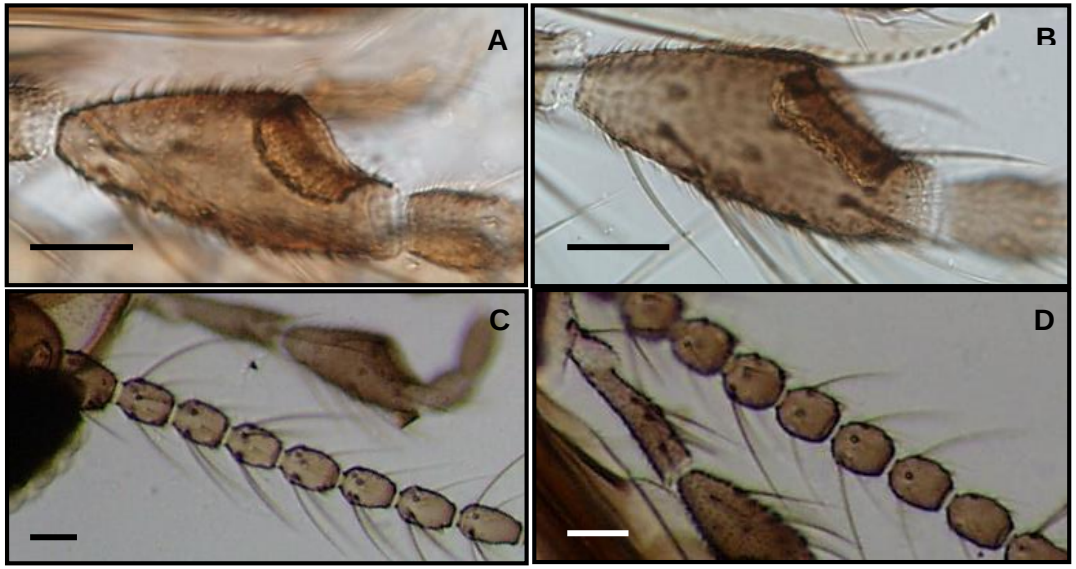


Culicoides semimaculatus has a strongly swollen 3rd palpus segment (A), spermathecae with a long pigmented neck and a donut shaped sclerotic ring (B) (white arrow). Scale bars: 20 µm.



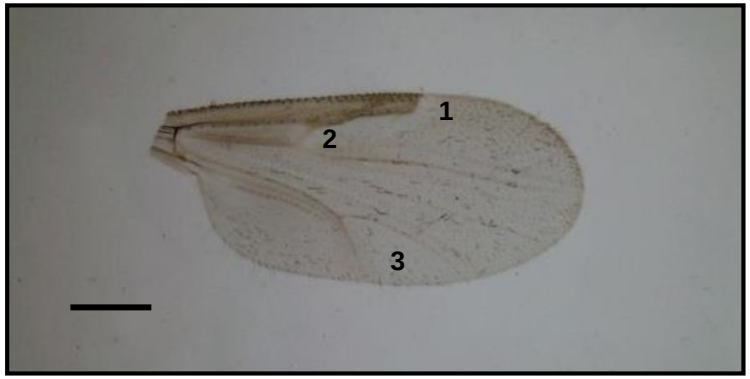
Culicoides subfasciipennis does not have a spine in the 4th tarsomere (C) and lacks sensilla coeloconica from the 5th to the 8th antennal flagellomere (D). Black arrows – tarsomere spines; 3 and 4 – 3rd and 4th tarsomere; 5 to 8 – 5th to 8th antennal flagellomeres. Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).



Culicoides heteroclitus and *Culicoides pseudopallidus* are very difficult to distinguish between them. 3rd palpus sensorial pit is shorter in *Culicoides heteroclitus* (A) than in *Culicoides pseudopallidus* (B). 2nd to 8th antennal flagellomeres may have an inflated shape in *Culicoides pseudopallidus* (D), although it can also have a flask shape like in *Culicoides heteroclitus* (C). *Culicoides heteroclitus* does not have a sclerotized ring in abdomen while in *Culicoides pseudopallidus* the sclerotized ring is present (not shown). Scale bars: 20 µm.

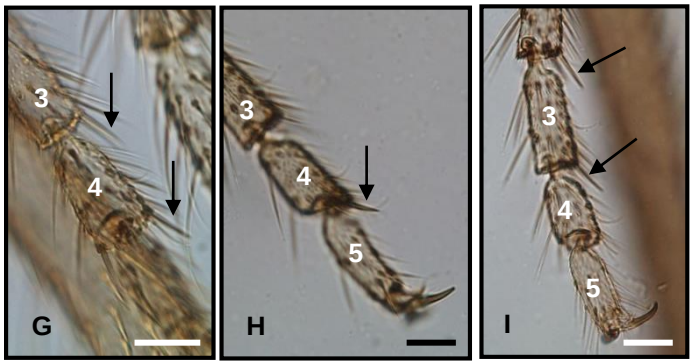
6. Dark wings with a very small poststigmatic pale spot (1) and a white spot in the r-m crossvein (2):



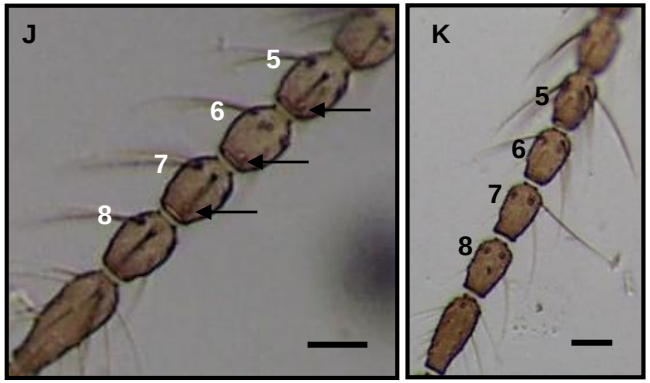
Includes: *Culicoides atripennis*, *Culicoides begueti* (left image), *Culicoides furcillatus*, *Culicoides indistinctus*, *Culicoides kibunensis*, *Culicoides odiatus* and *Culicoides pallidicornis*. *Culicoides kibunensis* can have an additional white spot in the cua_1 cell (3). Scale bar: 200 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

Third palpus segment of *Culicoides begueti* (A), *Culicoides indistinctus* (B), *Culicoides odiatus* (C), *Culicoides kibunensis* (D), *Culicoides pallidicornis* (E) and *Culicoides furcillatus* (F). All five species have different 3rd palpus segment shapes, being D, E and F more similar between them. Image F: Mathieu *et al*., 2012. Scale bars: 20 µm.



Both *Culicoides odiatus* (G), *Culicoides kibunensis* (H) and *Culicoides furcillatus* (not shown) have spines from the 1st to the 4th tarsomere, while *Culicoides pallidicornis* (I) have spines from the 1st to the 3rd tarsomere. Black arrows: tarsomere spines; 3 to 5: 3rd to 5th tarsomeres. Scale bars: 20 µm.



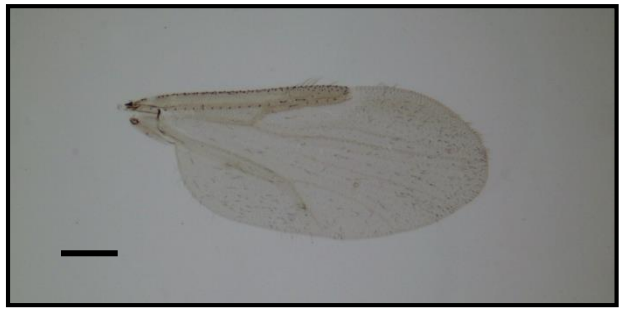
Culicoides kibunensis (J) has sensilla coeloconica from the 5th to the 8th antennal flagellomere (8th not shown), while *Culicoides pallidicornis* (K) does not have. Furthermore, *Culicoides furcillatus* has sensilla coeloconica on the 1st antennal flagellomere (not shown) while *Culicoides kibunensis* has sensilla coeloconica from the 1st to the 4th antennal flagellomere (not shown). Black arrows: sensilla coeloconica; 5 to 8: 5th to 8th antennal flagellomeres. Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

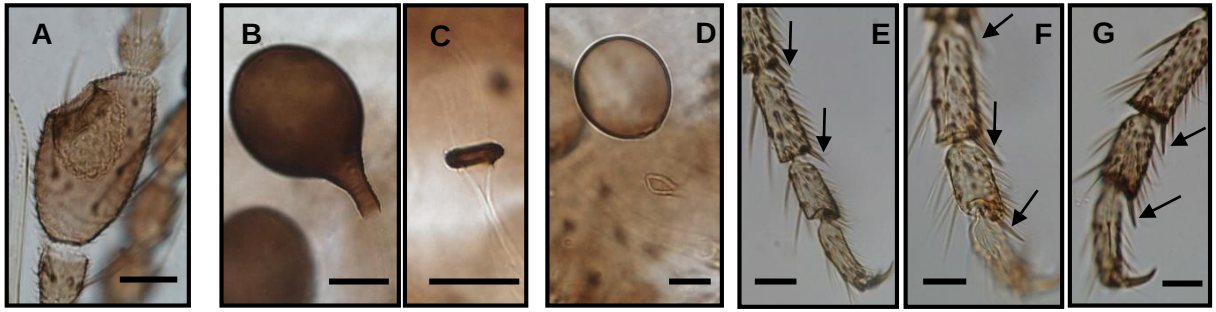
Note: *C. atripennis* and *C. kibunensis* differ only in body size, being the first species bigger than the second. The maximum length of some *C. kibunensis* body structures are as follows (Delécolle, 1985):

Body structure	Length (µm)
Wing	1206
Palpus	200
Antenna	622

7. Dark wing pattern without white spots:

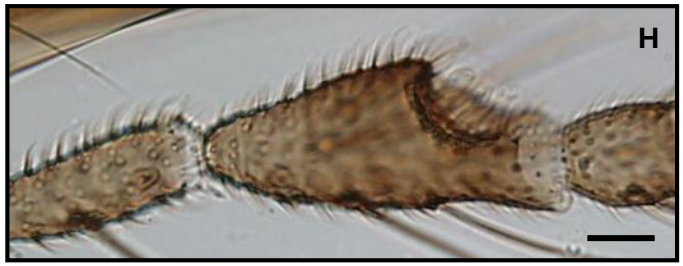


Includes: *Culicoides corsicus* (left image), *Culicoides derisor*, *Culicoides jurensis*, *Culicoides malevillei*, *Culicoides riebi* and *Culicoides tbilisicus*. Scale bar: 200 µm.



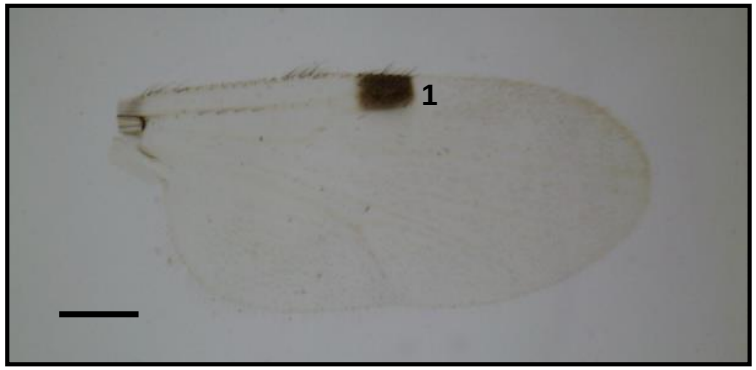
Culicoides jurensis (A) has a strongly swollen 3rd palpus segment. *Culicoides corsicus* (B and C) has a long spermatheca pigmented neck and its sclerotic ring has a donut shape. *Culicoides derisor* (D) does not have a spermatheca pigmented neck. *Culicoides malevillei* does not have a spine in the 4th tarsomere of middle legs (E), while *Culicoides riebi* (F) and *Culicoides tbilisicus* (G) have a spine in the 4th tarsomere of middle legs. Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

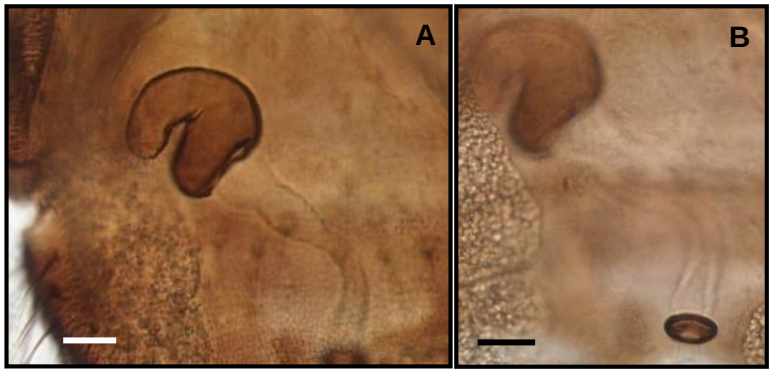


Culicoides riebi (H) has a single sensorial pit, while *Culicoides tbilisicus* (I) has multiple sensorial pits. Scale bars: 10 µm.

8. Light wing pattern with only one dark spot in the radial cells (1):



Although there are, at least, three species with this wing pattern in Palearctic ecozone (*Culicoides helveticus*, *Culicoides parroti* and *Culicoides stigma*), only *Culicoides parroti* (left image) was referred in Portugal and, thus, only that species is referred in this identification key. Scale bar: 200 µm.

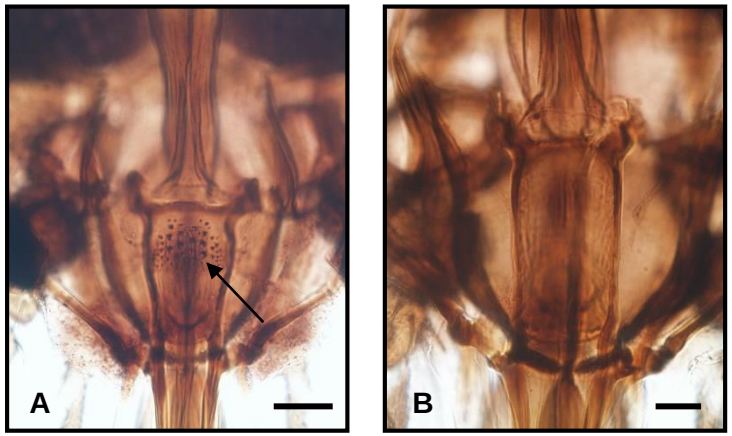


Culicoides parroti – This species has an “U” shaped spermathecae (A) and a donut shaped sclerotic ring (B). Scale bars: 20 µm.

Figure 2.12. – Identification key for Portuguese *Culicoides* female specimens (original photos, except where noted). (Continuation).

9. Light wing pattern with no dark spots:

Includes: *Culicoides heliophilus* and *Culicoides vexans* (left image). Scale bar: 200 µm.



Culicoides heliophilus (A) has an ornamented cibarium (black arrow), while *Culicoides vexans* (B) presents a simple cibarium. Scale bars: 20 µm.

2.5. Discussion

One of the aims of this study was to evaluate the distribution of different *Culicoides* species in Portuguese territory.

The relative frequency of captured *Culicoides* is completely different between species, ranging from 70.92% of *C. imicola* to <0.001% of *C. fagineus*, *C. kingi* and *C. paolae*. This extraordinary difference in relative frequencies may be due to different reasons:

1) *Culicoides* species preferences for different habitats (including environment, available hosts, etc.) may justify the quantity and diversity of *Culicoides* species collected with CDC and Onderstepoort light traps; as examples, most of the *C. paradoxalis* specimens were captured with swine farms nearby and *C. kingi* eggs and larvae have been found in ornamental plants transported between countries, as well as some species that are only found near wild animal fauna (Delécolle, personal communication, February, 2013).

2) The type of traps used during the NESP – although Onderstepoort traps may be more effective at collecting *Culicoides* (Venter *et al*., 2009), miniature CDC traps were used, because they are considered lighter and can be powered by a battery pack rather than requiring a main electric connection, making it easier to deploy in remote areas (Ramilo *et al*., 2012).

3) The spatial distribution of *Culicoides* species in mainland Portugal, which is also different, following some patterns. In this way, we observed 4 different types of distribution:

a) Species that are dispersed in all regions of mainland Portugal (*e.g.*, *C. achrayi*, *C. circumscriptus*, *C.festivipennis*, *C. punctatus*).

b) Species present mainly in north and central regions (*e.g.*, *C. albihalteratus*, *C. deltus*, *C. heliophilus*, *C. vexans*).

c) Species present mainly in central and south regions (*e.g.*, *C. nubeculosus*, *C. puncticollis*, *C. sahariensis*, *C. semimaculatus*).

d) Species consistently captured in specific regions (*e.g.*, *C. corsicus*, *C. impunctatus*, *C. lupicaris*).

Other species from Palearctic ecozone may have different hosts as favourite ones, being less or not captured at all by the traps used during the NESP and VectorNet European network. Other possible explanation is that those species do not make multiple generations, being uni- or bivoltine and living for a shorter period. Consequently, they might not live long enough to transmit diseases which they might carry.

The diversity of species captured in Madeira and Azores archipelago is low when compared with mainland Portugal (2 to 4 species against 64), probably because of the distance of the islands from the continent and the resulting low likelihood of *Culicoides* being introduced via wind transportation (Diaz *et al*., 2005; Ramilo *et al*., 2012). This hypothesis is supported by the fact that the number of *Culicoides* species present in the western islands of Azores archipelago (Flores and Corvo) is lower than the rest of the islands (2 species against 4).

These findings correspond entirely to what would be predicted by the theory of island biogeography (Ramilo *et al*, 2012). Another possible explanation for this small species diversity may be connected with the less captures performed in these archipelagos when compared with mainland Portugal.

The presence of species belonging to Obsoletus group in all islands of Madeira and Azores archipelago must be taken into account, since they are incriminated as potential vectors of BTV or SBV. This emphasizes the importance of veterinary education and surveillance measures to minimize the impact of future incursions of these diseases into these archipelagos.

During this study, the following species: *C. nubeculosus*/*C. puncticollis*/*C. riethi*, *C. parroti*/*C. stigma*/*C. helveticus* and *C. kibunensis*/*C. atripennis* were analysed as presented since these Palearctic species are morphologically indistinguishable when analysed with SM and have the same wing pattern.

C. nubeculosus, *C. puncticollis* and *C. riethi* species only differ between them by their spermatheca and spermathecal duct conformation. It was observed a discrepant distribution of the two identified species, *C. nubeculosus* and *C. puncticollis*, being the former present in only one of the analysed GUs. Thus, the idea that both species are equally distributed in one region or sample must not be followed as a rule. Moreover, it should be noticed that, although one species is not identified during specimens evaluation it does not necessary mean that that species is absent, what may possible be the case of *C. riethi*, since it was already mentioned in mainland Portugal by other authors (Cambournac, 1970b) and not all specimens were dissected and mounted for COM evaluation.

C. parroti only differ from *C. stigma* and *C. helveticus* by their spermathecae and sclerotic ring conformation. *C. helveticus* differ from *C. stigma* by the presence of sensilla coeloconica in the 1st and in the 3rd-8th antennal flagellomeres, while *C. stigma* only has sensilla coeloconica in the 1st and in the 6th-8th antennal flagellomeres. Only *C. parroti* was identified during specimen's analysis with COM, showing, once more, that similar species may not be equally distributed; however, the hypothesis that the other two species are absent from mainland Portugal must not be followed as a rule.

Finally, *C. atripennis* and *C. kibunensis* have the same wing pattern and only differ in their body size, being *C. atripennis* bigger than *C. kibunensis*. (Delécolle, personal communication, September 09, 2015). Both species were identified in this work, although with significant different proportions (4.76% for *C. atripennis* and 95.24% for *C. kibunensis*). Thus, specimens with *C. kibunensis*/*C. atripennis* characteristics must be always analysed with COM for a correct diagnose.

C. kingi is a recognized vector of Akabane disease and Epizootic Hemorrhagic disease and a potential vector of African Horse Sickness (Mellor, Osborne & Jennings, 1984; Fall *et al*., 2015). *C. kingi* species does not seem to be halophile, since it occurs far away from the

coast line (Cornet & Brunhes, 1994). However, its larval ecology is poorly known (Fall *et al*., 2015). *C. kingi* was referred for the first time in mainland Portugal in February 2013, GU 11, a place far away from the coast line, which is in agreement with Cornet & Brunhes (1994). Although only one male specimen was identified, this species must be taken into account when evaluating Portuguese *Culicoides* fauna.

Venail *et al*. (2012) mentioned the importance of surveillance measures and veterinary education that must be performed to detect the introduction of invasive species, the existence and circulation of a given pathogen in insect population and to follow up vector populations dynamics, in order to minimize the impact of these diseases in case of an outbreak.

Several *Culicoides* species are well adapted to mainland Portugal environment: *C. circumscriptus*, *C. fascipennis*, *C. gejgelensis*, *C. imicola*, *C. newsteadi*, specimens classified as *C. nubeculosus/C. puncticollis/C. riethi*, *C. pulicaris*, *C. punctatus*, *C. univittatus* and species belonging to Obsoletus group, since they appear all year in Portuguese territory, completing several generations in that same period (multivoltinism). *C. impuctatus* and *C. festivipennis* can also appear during 11 and 10 months, respectively. Furthermore, 40 of the referred species (75.47%) can persist during 6 or more months in mainland Portugal. The mean time of *Culicoides* species persistence in mainland Portugal is 7.23 months per year.

Seasonality of *Culicoides* species is of extreme importance, since it is one of the major factors related with the seasonality of diseases outbreaks (Howell, 1979; Herniman *et al*., 1983; Elfatih *et al*., 1987; Mohammed & Mellor, 1990; Baylis *et al*., 1997) and, together with parity status and daily survival, is an important factor to assess the potential for pathogen transmission in an area (Lysyk, 2007). Since the abovementioned species can persist in a region time enough to transmit pathogens that they might carry to susceptible hosts, further studies of vectorial competence and capacity should be performed in those species not incriminated as vectors of VBD. Parity status of *Culicoides* female specimens was not evaluated in this work, since it was not in the scope of the same.

The long persistence of some *Culicoides* species during the year probably occur due to the Portuguese climate, which is favourable to their occurrence, together with other biotic (*e.g.*, available hosts) and abiotic factors (*e.g.*, humidity, temperature and rainfall). Furthermore, this study also shown that up to 35 different *Culicoides* species can occur at the same sampling place (GU 26, performed captures: 115), followed by 33 (GU 40, performed captures: 104), 31 (GU 4, performed captures: 80) and 30 (GU 36, performed captures: 75) species in different regions of mainland Portugal (North, Centre and South), which is in agreement with Blackwell *et al*. (1992b). These results show a little bit more diversity of *Culicoides* species occurring at the same trap site than that shown by Scolamacchia *et al*. (2014) (n=22). Finally, a mean of 8.75 different species occurred in each sampling place in mainland Portugal.

Further studies must be performed in capture sites where a higher number of *Culicoides* species were observed and a small number of captures were performed (*e.g.*, UG 30, with 29 different species in 36 performed captures) and vice-versa (*e.g.*, UG 8, with 6 different species in 104 performed captures) to understand the biotic and abiotic factors that might favour or disfavour *Culicoides* species occurrence, respectively.

Although four mainland Portugal GUs were not sampled (2, 41, 43 and 45), since they were not considered to be of epidemiological interest due to low livestock densities, captures must be performed in these regions near other species (*e.g.*, wild ruminants) in order to understand *Culicoides* species presence/absence and their distribution in those areas.

According to Garros *et al.* (2010), molecular data cannot replace classical taxonomy, since these methods are contributing to the disappearance of morphological taxonomic expertise (Wilson, 1985), in a so called "taxonomic impediment" (Tautz, Arctander, Minelli, Thomas & Vogler, 2003). An "integrative" future for *Culicoides* taxonomy should be promoted, where molecular, ecological and morphological analysis are closely linked (*e.g.*, Holbrook *et al*., 2000; Meiswinkel & Linton, 2003).

Microscopic (SM, COM and SEM) and molecular techniques (PCR) were used in this work, in collaboration with FCUL, CIRAD (Montpellier) and University of Strasbourg, for an accurate determination of *Culicoides paradoxalis* as a valid species inside *Culicoides* subgenus. It was observed that *C. newsteadi* was morphologically closer to *C. paradoxalis* than to *C. lupicaris* (mainly because of their wing pattern); however, *C. lupicaris* is molecularly closer to the new species than *C. newsteadi* (mean distance of the COI region: 0.166 and 0.210, respectively). According to Darwin's theory (Darwin, 1859), *C. paradoxalis* and *C. lupicaris* may had suffer of divergent evolution, *i.e.*, both species came from a common ancestor (high genetic similarity) but in order to adapt to different niches they evolve differently (low phenotypic similarity). On the other hand, *C. paradoxalis* and *C. newsteadi* may had suffer of convergent evolution, *i.e.*, these two species, without a common ancestor (low genetic similarity), were submitted to similar niches and during evolution they converged (high phenotypic similarity).

This situation shows the importance of an integrative taxonomy approach, which must be performed whenever possible in order to understand different *Culicoides* species position inside taxonomy and relation between closer species. Furthermore, intensive studies with species belonging to *Culicoides* subgenus must be performed, since cryptic diversity was observed within *C. pulicaris* clade, as mentioned in previous works (Pagès *et al*., 2009) and, as *C. pulicaris* is a vector of BTD, others inside the same genus may be also vectors of the same and/or other VBD.

Besides the first reference of *C. paradoxalis* in France and Portugal (Ramilo *et al*., 2013), this species was also referred in 2014 in Spain, in Extremadura Autonomous Community, between June and October (Sánchez Murillo, González, Martínez Díaz, Reyes Galán &

Alarcón-Elbal, 2015). In mainland Portugal, this species was detected during a longer period, namely January, March and May to November. These different results may be due to the extension of geographical areas and time covered in both works and, so, further efforts must be done to investigate this species dispersion and prevalence in Spain, as well as in other countries.

Another aim of this study was to describe *Culicoides* fauna in Portuguese territory. After evaluation of collections made during the NESP and VectorNet European network, *Culicoides* species referred in mainland Portugal raised from 47 to 64 species, although 8 of the species referred by other authors from the 1952-2005 period were not observed. In Azores archipelago, the number of *Culicoides* species identified raised from one to four. In Madeira archipelago, all species previously identified by other authors (Capela *et al*., 1990, 1997) were captured during the NESP, with the exception of *C. puncticollis* in Porto Santo island.

C. almeidae can be easily identified as *C. punctatus* by the existing photographs of its wing pattern (Delécolle, personal communication, July 20, 2015), being a synonym of this species. Concerning *C. nuntius* and *C. rochenus*, their brief description is not enough to evaluate correctly if they are effectively independent species or synonymies of existent ones. Lamblé *et al*. (1990) reported for the first time in Portugal *C. ribeiroi*, suggesting it was a new species for science; however, *C. ribeiroi*, together with *C. dendriticus*, are synonymies for *C. tbilisicus*, described for the first time by Dzhafarov (1964a).

Other specimens described by Pena (2003) and Vila-Viçosa *et al*., (2009) have morphological characteristics similar to other *Culicoides* species. These specimens were reviewed after using Mathieu *et al*., (2012) interactive identification key for female *Culicoides* biting midges.

C. jumineri specimens classified by Pena (2003) and Vila-Viçosa *et al*. (2009) possess morphological characteristics similar to *C. jumineri* near *C. bahrainensis* instead, since those specimens have both characteristics of these two species (Delécolle, personal communication, March 22, 2010). Also, photographed *C. brunnicans* specimens have morphological characteristics similar to *C. santonicus* instead.

Pena (2003) classified one female specimen as *C. clintoni*. However, after using Mathieu *et al*. (2012) interactive identification key, together with morphological characteristics described and photographs obtained by Pena, it is valid to say that the referred specimen is a *C. albihalteratus*. Similarly, morphological features of *C. truncorum* pointed out by Pena (2003) are more characteristic of *C. gejgelensis* after using Mathieu *et al*. (2012) interactive key.

Additionally, some specimens point out by Vila-Viçosa *et al*. (2009) should be renamed: *C. jurensis* as *C. indistinctus*, *C. impunctatus* as *C. fagineus* and *C. subfagineus* (two different specimens), *C. duddungstoni* as *C. festivipennis*, and *C. marcleti* as *C. corsicus*. The specimen identified as *C. heliophilus* belongs to other species due to its morphological

characteristics (Mathieu *et al*., 2012); however, available photographs are not enough to identify the specimen species. Finally, Vila-Viçosa *et al.*, (2009) observed a *C. montanus* male specimen but did not mention it as belonging to this species.

The raise in the number of *Culicoides* species observed in Portugal may be due to the global warming and adaptation of these biting midges to our environment and climate after their introduction in mainland Portugal by wind or by human intervention (*e.g.*, planes, boats or animal movements). However, some of the species referred in mainland Portugal for the first time were already been detected by previous works but were mistakenly identified (*e.g.*, *C. santonicus*, *C. jumineri* near *C. bahrainensis*, *C. subfagineus*). So, another reason, such as lack of information about *Culicoides* identification in available identification keys may be subjacent to these errors, together with some species that were not caught in mainland Portugal and classified as been (*C. clintoni*, *C. duddingstoni, C. marcleti* and *C. truncorum*) or others classified as new species when, in fact, they have been identified for the first time by other authors (*C. ribeiroi*).

With new and more easy accessible interactive keys (Mathieu *et al*., 2012), taxonomical classification of this genus has permitted more certainties when evaluating *Culicoides* specimens and their identification to species. These keys are of a crucial importance, since their use can avoid errors during classification of *Culicoides* species by taxonomists.

Previous studies conducted in Portugal made use of small collections when comparing to those achieved in the NESP for BTD during an 8 year period and some of the *Culicoides* species found since 2005 may not be caught before, although they have already been in Portuguese territory for a long time. However, *Culicoides* species mentioned before 2005 and not captured during NESP have a higher probability to be absent in mainland Portugal, due to the extension (both spatial and temporal) of the surveillance program. Finally, the capture of *C. paolae* only during VectorNet European network may be due to the type of trap used, since Onderstepoort black-light traps have a more powerful light source and fan and, for this reason, they can collect significantly more *Culicoides* midges under field conditions than other kinds of traps (Venter *et al*, 2009; Venter, Majatladi, Labuschagne, Boikanyo & Morey, 2012). The specimen captured was an engorged female with eggs, which is a good indicative that this species is probably established in the region where it was collected (GU 23) and its presence and capture was not accidental (*e.g.*, carried by the wind).

Although some species appeared only in horse farms (*C. corsicus*, *C. fascipennis*, *C. haranti*, *C. odiatus*, *C. santonicus* and *C. tbilisicus*) while others appeared only in cattle farms (*C. derisor*, *C. lupicaris*, *C. paolae* and *C. puncticollis*), the number of *Culicoides* species captured near cattle and horses during VectorNet field work (2015) was almost the same (26 and 27 in cattle and horse farms, respectively). These preliminary results should be further evaluated using molecular techniques to characterize feeding preferences, since the presence of different *Culicoides* species in traps does not mean that they perform their blood

meal on the most near host (Dzhafarov, 1964a; Scheffer *et al*., 2012; Elbers & Meiswinkel, 2014). Moreover, different ecological conditions could have influenced these results and, thus, this study should be performed in other regions of mainland Portugal to confirm or not these data.

Culicoides biting midges play an important role as vectors of different diseases, causing morbidity and mortality in affected animals, as well as huge economical losses to the affected countries. Taxonomic classification of *Culicoides* species is of extreme importance since the phenotypic and genetic traits of vector species play a key role in determining the epidemiology of diseases transmission. Subtle differences in the biology and ecology of closed related species can exert significant effects on the probability of transmission, being vector competence and host preference the most important factors (Harrup *et al*., 2014).

The taxonomic identification key was elaborated using photographs from different *Culicoides* anatomical structures, for an easy and faster laboratorial identification of the different *Culicoides* species that can appear in Portuguese territory. This procedure is of special scientific and pedagogical importance, aiming to support students and future researchers in this area. However, this key should be used with caution, since it does not refer species that were not captured during the NESP for BTD and VectorNet European network. As example, *C. parroti*, *C. stigma* and *C. helveticus* must be classified altogether when based exclusively in wing pattern, because, although two of them were not observed during *Culicoides* laboratorial identification, all specimens must be slide-mounted for posterior observation with COM, for they can be *C. helveticus* or *C. stigma*.

Concerning vector-borne diseases, vectors have an important role in the so-called epidemiologic triad, since they are responsible for the transmission of the pathogen to a susceptible host. Thus, a thorough knowledge of the vector and the factors that control their appearance, development, prevalence and death are necessary to fully understand the dynamics of VBD. Analysing *Culicoides* fauna present in a specific area, *Culicoides* species distribution, seasonal occurrence, among other factors, is of critical importance, since this information can contribute to understand the processes by which a disease appears, maintains and spreads from an area or region. Thus, this work gives an important overview about Portuguese *Culicoides* fauna since 1952 until nowadays, revealing a new species for science, *C. paradoxalis*, which belongs to *Culicoides* subgenus (that includes *C. pulicaris*, a BTD vector species), and giving origin to an important identification key for female *Culicoides* present in Portuguese territory, which can be used with both educational and research proposes.

Chapter 3: Distribution of different species within Obsoletus group in mainland Portugal (2006-2009)

3.1. Introduction

Species within Obsoletus group are extremely important in Veterinary Medicine, since they are known vectors of several animal viruses, like AHSV, BTV, EHDV and SBV (Table 1.1., page 4). Most of the species belonging to *Culicoides* genus can be easily identified using their wing pattern (Rawlings, 1996). However, female midges belonging to Obsoletus group have the same wing pattern and thus their differentiation must rely on other morphological aspects (Delécolle, 1985; Mathieu *et al*., 2012).

Within Obsoletus group, females of *C. chiopterus* can be distinguished by the presence of interfacetal hairs, while *C. dewulfi* has two different sized spermathecae (Delécolle, 1985; Mathieu *et al*., 2012).

C. obsoletus, *C. scoticus* and *C. montanus* form the Obsoletus complex, since females of these species are very difficult to distinguish between each other (Monaco *et al*., 2010; Sarvašová, Goffredo, Sopoliga, Savini & Kočišová, 2014). Some works refer the format and the ratio length/width (L/W) of the 3rd palpus segment, as well as its sensorial pit depth, wing geometry, morphological conformation of abdomen chitinous plates (parallel or convergent) and spermathecae size as useful characteristics to distinguish these three species (Delécolle, 1985; Nielsen & Kristensen, 2011; Mathieu *et al*., 2012; Hajd Henni *et al*., 2014; Kirkeby & Dominiak, 2014). Until 2012, only three species from Obsoletus group (*C. obsoletus*, *C. scoticus* and *C. chiopterus*) were identified in Portuguese territory (Pena, 2003; Ramilo *et al*., 2012).

According to some authors, there are several factors affecting the distribution of *Culicoides* species, being the climate (primarily rainfall and temperature) the most influent one in seasonal dynamics of these midges (Venail *et al*., 2012).

Mainland Portugal climate can be defined as dry climate with hot summers (usually known as Mediterranean or Csa climate) in the regions below the 39.66025 N latitude approximately (including Beira Baixa region), and as dry climate with warm summers (usually known as Csb climate) in the regions above the same mentioned latitude, according to the Köppen-Geiger Climate Classification (Peel, Finlayson & McMahon, 2007; Brugger & Rubel, 2013).

3.2. Objectives

The aims of this study were:

1) Identify Obsoletus group specimens to species level, during a four year period (2006-2009) in 16 different farms, 3 to 5 for each different region of mainland Portugal (North, Centre North, Centre South and South) in order to understand their distribution.
2) Analyse the influence of two abiotic factors (temperature and rainfall) in the presence or absence of species inside Obsoletus group per season.
3) Evaluation of possible anatomic aberrations of species belonging to Obsoletus group.

4) Evaluation of a more accurate cut-off value of the 3rd palpus segment L/W ratio to identify different species inside Obsoletus group.

3.3. Materials and methods

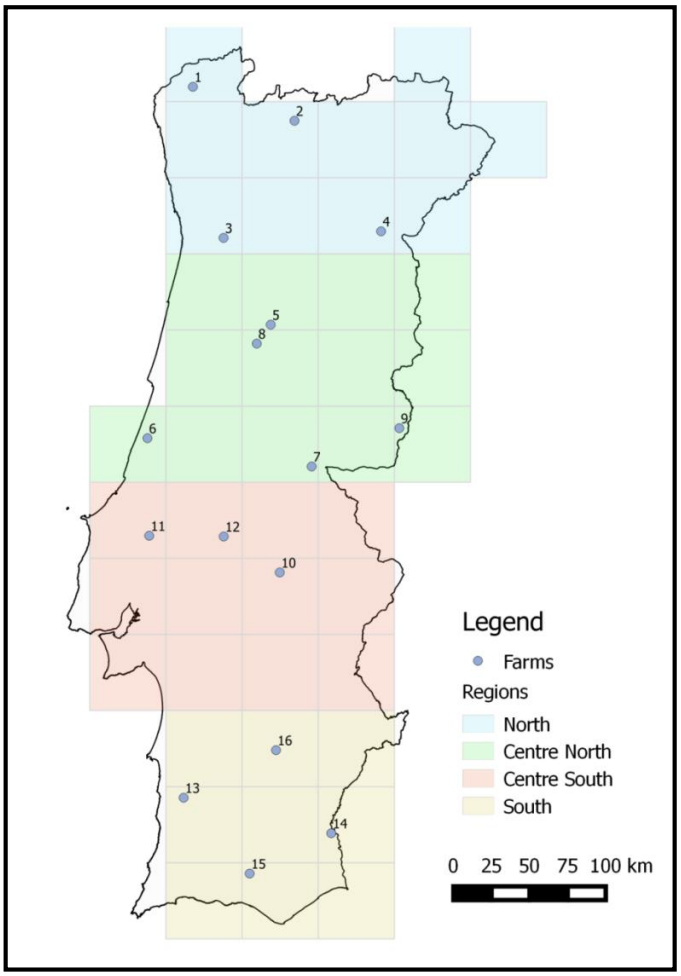
3.3.1. NESP for BTD and insect sampling

Culicoides biting midges were captured in the scope of the NESP for BTD as described in Chapter 2 (see 2.3.1. and 2.3.2.). Sometimes, the periodicity of insect sampling was changed due to Veterinary services decision, trap technical problems or adverse meteorological conditions.

3.3.2. Farm selection and abiotic factors analysis

For this study, mainland Portugal was divided into 4 different regions: North (from GU 1 to 11, blue squares), Centre North (from GU 12 to 24, green squares), Centre South (from GU 25 to 36, pink squares) and South (from GU 37 to 45, brown squares) (Figures 2.1. [page 62] and 3.1.). From within each region, a maximum of three to five farms with the highest number of performed captures per GU were selected for this study (Figure 3.1.).

Figure 3.1. – Division of mainland Portugal into 4 regions and localization of selected farms.



All selected farms belonged to a different GU. The geographic coordinates of each one are shown on Table 3.1, as well as the distance of each selected farm to the closest meteorological station.

Table 3.1. – Geographic coordinates of selected farms and their distance in straight line to the closest meteorological station.

Farm identification	Region of Mainland Portugal	Geographic coordinates		Distance to the closest meteorological station (km)
		Latitude (N)	Longitude (W)	
1	North	41.9168	-8.5709	36.85
2	North	41.7162	-7.7677	49.38
3	North	41.0235	-8.3252	37.99
4	North	41.0580	-7.0946	57.46
5	Centre North	40.5102	-7.9584	19.59
6	Centre North	39.8359	-8.9060	51.25
7	Centre North	39.6698	-7.6472	46.02
8	Centre North	40.3983	-8.0667	39.65
9	Centre North	39.8921	-6.9725	57.96
10	Centre South	39.0433	-7.8933	49.57
11	Centre South	39.2585	-8.8861	46.32
12	Centre South	39.2563	-8.3193	77.62
13	South	37.7092	-8.6139	33.69
14	South	37.4981	-7.5161	66.29
15	South	37.2610	-8.1220	30.5
16	South	37.9917	-7.9250	6.27

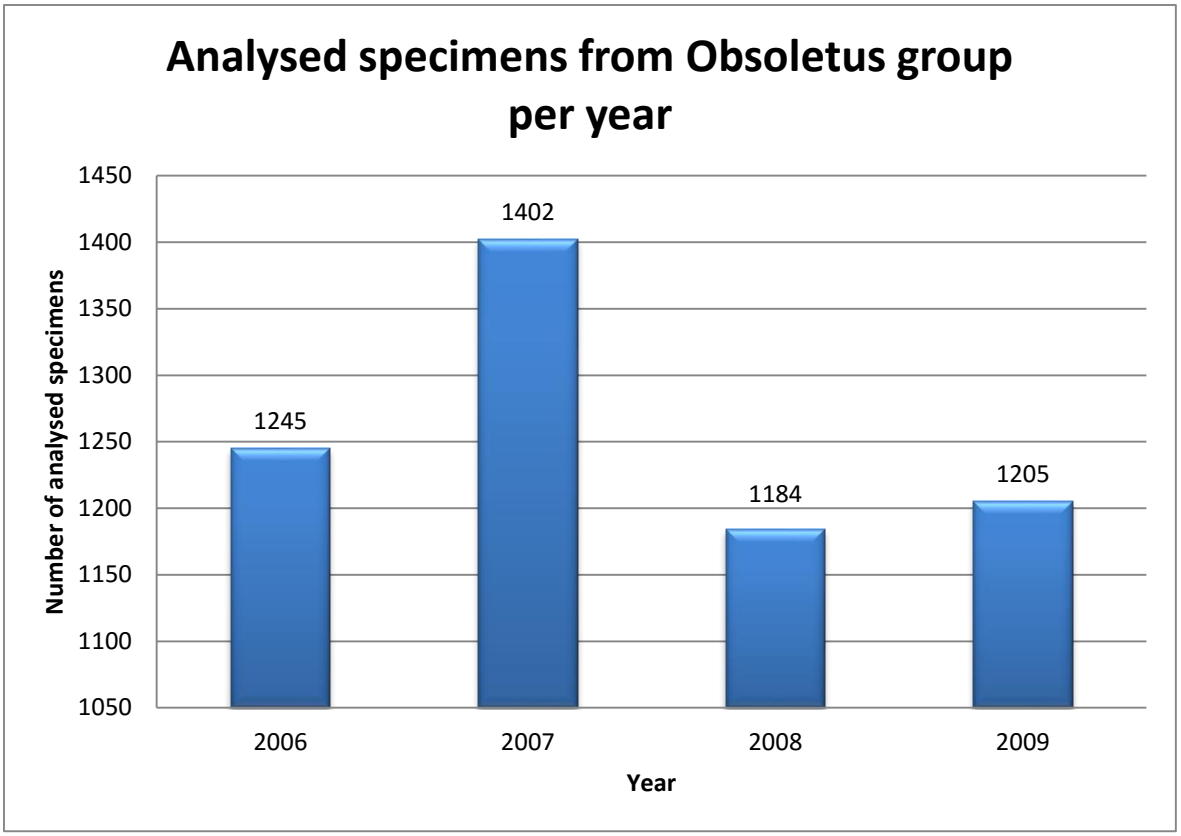
Monthly, minimum and maximum mean temperatures for each region were obtained from Instituto Português do Mar e da Atmosfera (IPMA) meteorological stations closest to the selected farms. In addition, meteorological bulletins made by IPMA were also analysed together with Obsoletus group species occurrence (IPMA, 2015).

Seasons were the period of time considered for this study: spring (March, April and May), summer (June, July and August), fall (September, October and November) and winter (December, January and February).

3.3.3. Sample selection and morphological identification

A total of 5 036 specimens were analysed morphologically (corresponding to 7.07% of the analysed individuals belonging to Obsoletus group during 2005-2013 NESP period), with their distribution shown in Figure 3.2..

Figure 3.2. – Annual distribution of analysed specimens from Obsoletus group.



The identification of *Culicoides* genus was performed using SM together with identification keys (Delécolle, 1985; Mathieu *et al*., 2012).

For the 2006-2009 period a maximum of 8 females were randomly selected per capture and were dissected into 4 different body parts (head, thorax with legs, abdomen and wings) using 26 Gauge (0.404 mm diameter) needles. Thorax, legs and one wing were stored in 96% ethanol for further molecular biology analysis by conventional PCR. Head, abdomen and the other wing were mounted on glass slides using Hoyer's medium and dried in an incubator at 37 °C for 3-4 days.

Specimens were then observed using COM for species identification and the following data were registered (Delécolle, 1985; Nielsen & Kristensen, 2011; Mathieu *et al*., 2012):

1) 3rd palpus segment L/W ratio, based on Nielsen & Kristensen (2011) work intervals (Table 3.2.).
2) The length of the two spermathecae, being this last feature the major characteristic used for species diagnosis, based on Delécolle (1985) work intervals (Table 3.2.):

Table 3.2. – 3rd palpus segment L/W ratio, as performed by Nielsen & Kristensen (2011) and spermathecae size, as performed by Delécolle (1985).

***Culicoides* species**	**Length/width ratio**	**Spermathecae size (µm)**
C. obsoletus	< 2.6	46-59
C. scoticus	> 2.7	57-67
C. chiopterus	< 2.5	46-52
C. dewulfi	> 2.7	1st: 40-44 2nd: 55-59

3) Morphological characteristics and sensorial pit depth of the 3rd palpus segment.
4) Morphological characteristics of the chitinous plates (parallel or convergent);
5) Presence/absence of pubescence between ommatids.

When males were present, a maximum of 8 specimens were identified to species by analysis of their genitalia structures (aedeagus, parameres, gonocoxite, gonostylus, 9th sternite, lamellae and basal membrane) (Delécolle, 1985; Mathieu, 2011; Nielsen & Kristensen, 2011).

All anatomical aberrations found in females were also registered.

3.3.4. Statistical analysis

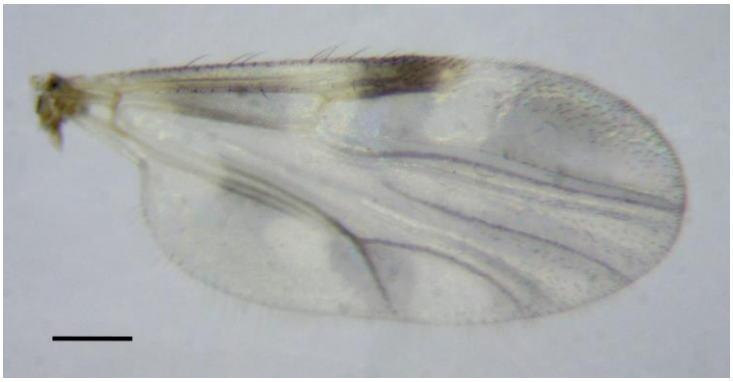
With the ratios L/W of the 3rd palpus segment obtained from *C. obsoletus* and *C. scoticus* species, a receiving operating curve (ROC) was constructed and a cut-off value (as well as its sensitivity and specificity) was obtained, based on the work of Bewick, Cheek & Ball (2004), with the R Studio® software. Furthermore, our cut-off value was compared with the previous value of 2.6 for *C. obsoletus* as determined by Nielsen & Kristensen (2011).

3.4. Results

3.4.1. Species identification

All *Culicoides* specimens with a wing pattern similar to that shown on Figure 3.3. were classified as belonging to Obsoletus group.

Figure 3.3. – Wing pattern of Obsoletus group species.



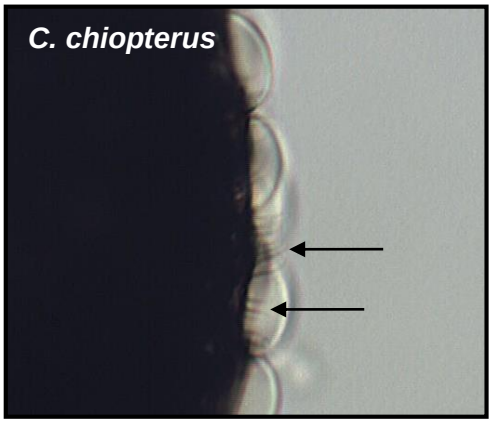
Scale bar: 200 µm. Original photo.

3.4.1.1. Females

During female specimens evaluation using COM, the following anatomic characteristics were observed, in this same order, to define each species inside Obsoletus group:

1) Observation of pubescence between ommatids: *C. chiopterus* is the only species inside Obsoletus group with this anatomical feature (Figure 3.4.).

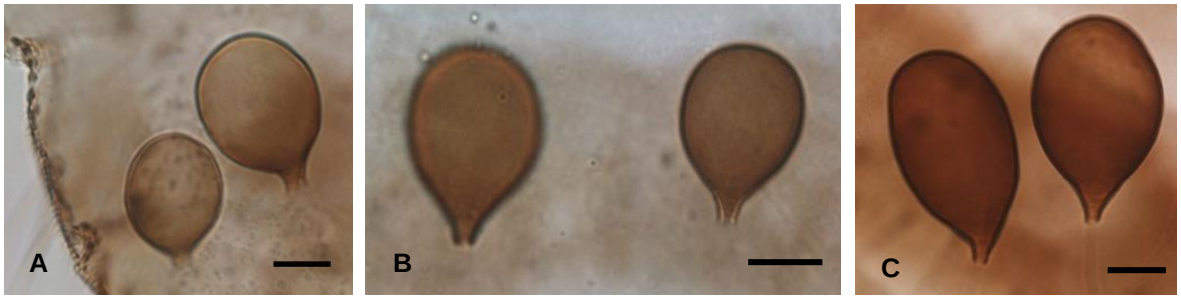
Figure 3.4. – Pubescence between ommatids (Mathieu *et al*., 2012).



Black arrows: pubescence between ommatids.

2) Measure of spermathecae size: *C. dewulfi* can be identified by the presence of two different sized spermathecae (Figure 3.5. A). In the presence of two equalled sized spermathecae, their length was obtained and compared for identification of *C. obsoletus* and *C. scoticus* (Figure 3.5. B and C).

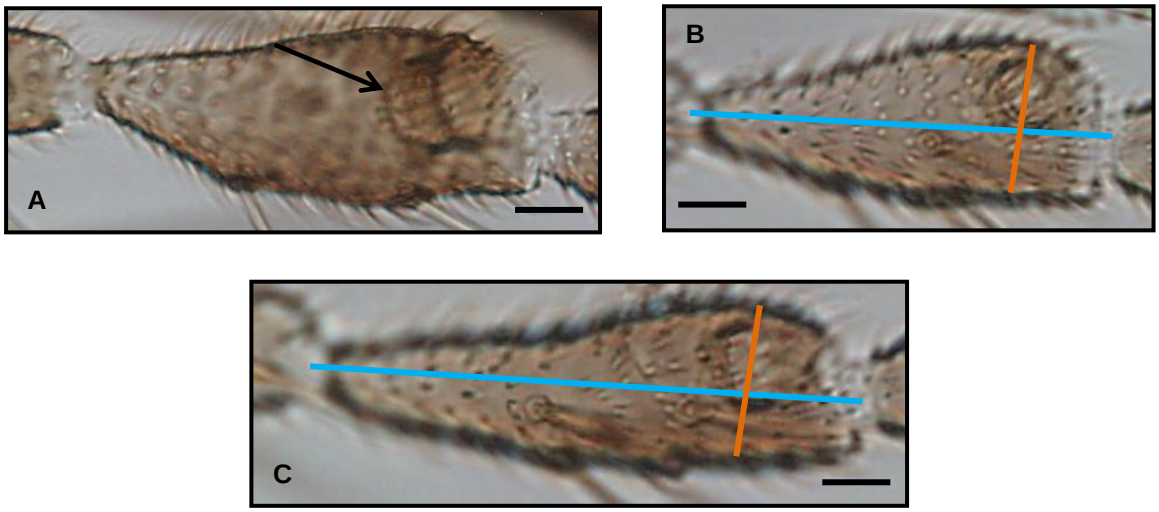
Figure 3.5. – Spermathecae of three Obsoletus group species.



A – Different sized spermathecae of *C. dewulfi*. *C. obsoletus* spermathecae (B) are smaller than *C. scoticus* spermathecae (C). Scale bars: 20 µm. Original photos.

3) Evaluation of the 3rd palpus segment conformation and sensorial pit depth: *C. montanus* can by identified by a deeper sensorial pit when compared with *C. obsoletus* and *C. scoticus* (Figure 3.6. A). Furthermore, the ratio L/W of the 3rd palpus segment was also evaluated to distinguish *C. obsoletus* and *C. scoticus* species (Figure 3.6. B and C).

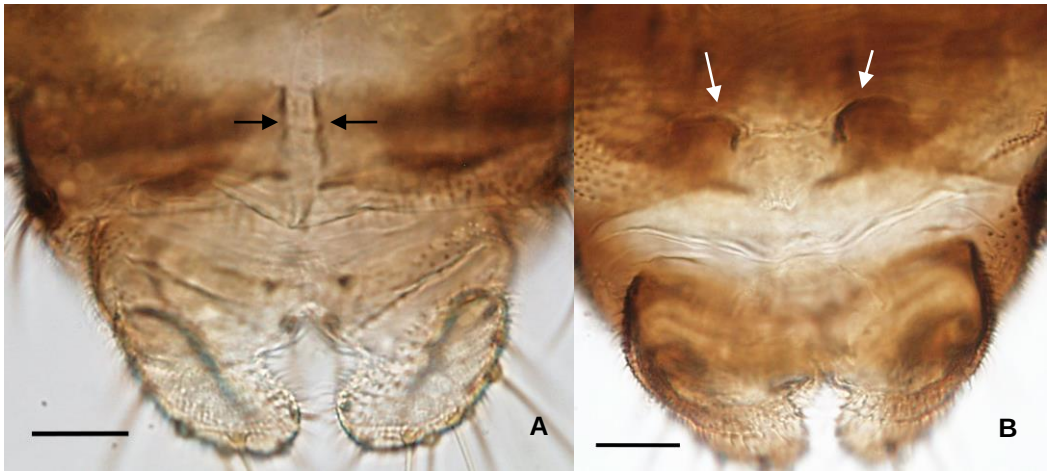
Figure 3.6. – 3rd palpus segment conformation and sensorial pit depth of species belonging to Obsoletus complex.



A – *C. montanus*; B – *C. obsoletus*; C – *C. scoticus*. 3rd palpus segment conformation is very similar between the first two species, differing only by the sensorial pit depth (balck arrow). Length and width is represented by the blue and orange lines, respectively. Scale bars: 10 µm. Original photos.

Additionally, the conformation of the chitinous plates (as parallel or convergent) of *C. obsoletus* and *C. scoticus* was also registered (Figure 3.7.).

Figure 3.7. – Chitinous plates conformation: parallel (A) and convergent (B).

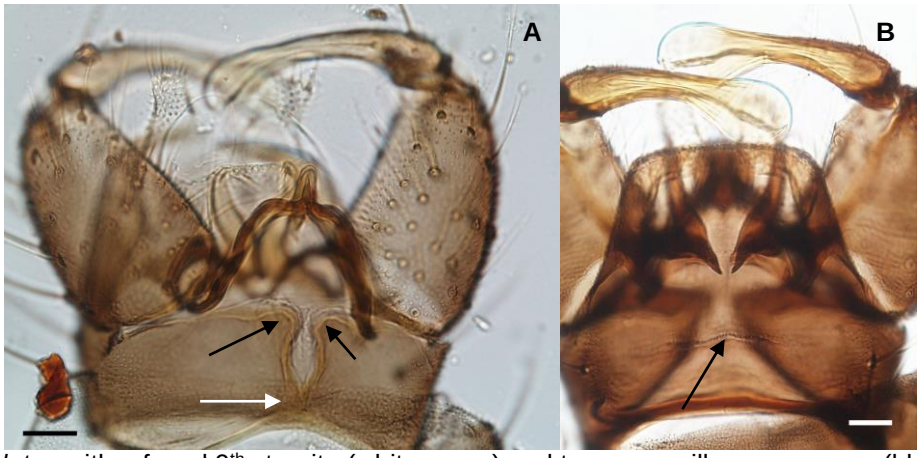


Black arrows: Parallel chitinous plates; White arrows: convergent chitinous plates.
Scale bars: 20 µm. Original photos.

3.4.1.2. Males

During male specimens evaluation using COM, the genital structures were observed to define each species inside Obsoletus group (Figure 3.8.).

Figure 3.8. – Genital structures of *C. obsoletus* and *C. scoticus* males.



A – *C. obsoletus* with a fused 9th sternite (white arrow) and two mammillary processes (black arrows). B – *C. scoticus* without a fused 9th sternite (black arrow). Scale bars: 20 µm. Original photos.

3.4.1.3. *Culicoides* spp. specimens

Culicoides spp. specimens (n=245; 4.95%) included those that could not be identified due to:

1) Absence of spermathecae.

2) 3rd palpus segment L/W ratio <2.6 and spermathecae size >57 µm.

3) 3rd palpus segment L/W ratio >2.7 and spermathecae size <59 µm.

4) Bigger spermathecae >59 µm and smaller spermathecae <57 µm.

Farms 2 (n=83), 1 (n=68) and 6 (n=31) were the ones with the biggest number of undiagnosed specimens, corresponding to 74.29% of the total.

3.4.2. Distribution of Obsoletus group species in mainland Portugal

The relative frequency of Obsoletus group midges per region of mainland Portugal is referred in Table 3.3.

Table 3.3. – Relative frequency of Obsoletus group midges per region of mainland Portugal (2006-2009).

Year	Region of Mainland Portugal (%)				Total (%)
	North	Centre North	Centre South	South	
2006	38.11	37.42	10.18	14.29	100
2007	36.11	35.87	12.38	15.63	100
2008	38.53	22.39	16.59	22.49	100
2009	42.73	28.86	3.13	25.28	100
Mean per region	38.87	31.14	10.57	19.42	100

The relative frequency of each Obsoletus group species per region of mainland Portugal is referred in Table 3.4.

Table 3.4. – Relative frequency of each Obsoletus group species per region (2006-2009).

Region of Mainland Portugal	*Culicoides* species (%)				Total (%)
	C. obsoletus	*C. scoticus*	*C. montanus*	*C. dewulfi*	
North	44.68	24.99	29.74	0.59	100
Centre North	34.56	35.89	29.55	0	100
Centre South	53.20	12.72	34.98	0	100
South	43.07	25.04	31.53	0	100

To the best of our knowledge, this work mentions for the first time the presence of *C. montanus* and *C. dewulfi* (syn.: *C. pseudochiopterus*) in mainland Portugal, raising to 66 the number of *Culicoides* species referred in Portuguese territory.

C. obsoletus specimens were found in the 16 selected farms, being the most prevalent species in North, Centre South and South regions of mainland Portugal (Table 3.4.). *C. scoticus* and *C. montanus* species were found in 15 of the 16 selected farms, being the former the most prevalent species in the Centre North region, but with very similar relative frequencies comparing to *C. obsoletus* (Table 3.4.). *C. dewulfi* was found in only three farms, all of them located in the North region. *C. chiopterus* was not found in any of the selected farms during specimens' morphological analysis.

The distribution of different species from Obsoletus group in Mainland Portugal is represented in Table 3.5..

Table 3.5. – Distribution of species from Obsoletus complex per region of mainland Portugal in 2006-2009 period.

Culicoides species	Region of Mainland Portugal (%)				Total (%)
	North	Centre North	Centre South	South	
C. obsoletus	25.66	22.09	25.59	26.66	100
C. scoticus	32.99	42.31	6.08	18.62	100
C. montanus	21.17	20.98	26.09	31.76	100

Months and regions where no midges belonging to Obsoletus group were caught are referred in Table 3.6..

Table 3.6. – Regions where no midges from Obsoletus group were captured per month.

Region of Mainland Portugal	Month	Year
Centre South	April, August	2006
South	August	
Centre South	November, December	2007
South	January	
Centre North	November	2008
Centre South	February, March, August, September, December	
South	January	

Table 3.6. – Regions where no midges from Obsoletus group were captured per month (Continuation).

Region of Mainland Portugal	Month	Year
Centre South	April, July, August, September, November, December	2009
South	June	

No captures were performed in December 2006 in North Region, January 2009 in Centre North region and in December 2006, January 2008 and 2009 in Centre South region due to Veterinary services decision, trap technical problems or adverse meteorological conditions. Bisides, no captures were performed on farm 8 (2006 and 2007), 9 (2006, 2007 and 2009), 11 (2006), 12 (2009) and 16 (2006 and 2007) due to Veterinary services decision.

3.4.3. Abiotic factors

After meteorological bulletin analysis, per season (IPMA, 2015), the following observations were registered:

1) Many sequenced days with very low temperatures (frost days) were registered in the winter of 2006 (January and February). In 2006/07, heavy rainfall was observed in February, together with mild temperatures. The winter of 2007/08 presented very hot and dry months, while the winter of 2008/09 had very cold days and nights. However, there was a regular rainfall during the three winter months in all mainland Portugal in 2008/09.

2) Concerning spring, the months of March and April 2007 were dryer than in 2006. April 2007 was extremely dry in the North, Centre and South (Algarve) regions, while in 2006 this month was rainy. April 2008 registered a level of precipitation higher than the normal, but in 2009 less precipitation was observed, with predominance of dry and hot weather.

3) Although rainy, summer 2006 presented very high temperatures, with four heat waves. The same period of 2007 was also rainy but milder temperatures were registered than in the same period of 2006. In summer 2008 no heat waves were registered, with low rainfall in both North and Centre North regions. In 2009, two heat waves were reported in the Northern inland regions of mainland Portugal and three heat waves occurred in Centre South and South regions.

4) In fall 2006 higher temperatures and rainfall than the same period of 2007 were recorded. However, in September 2007 high precipitation in Centre and South regions was registered. October and November 2008 were extremely dry months in Centre North and Centre South areas. However, rainfall was observed in North and South regions. In November 2009, there was heavy rainfall in North and Central regions. In Centre South region there were higher temperatures, with two heat waves, as well as low precipitation.

Seasonal characterization of mean air temperature and precipitation for mainland Portugal during the 2006-2009 period is represented in Table 3.7.

Table 3.7. – Seasonal characterization of mean air temperature and precipitation in mainland Portugal (2006-2009) (IPMA, 2015).

Year	Season	Mean Air Temperatures (Mean values) (ºC)[1]	Precipitation (mm)[1]	Observations
2006	Winter	8.26	187.92	Very dry season
	Spring	14.99	182.5	Dry season; March very rainy
	Summer	22.76	68.5	5th hottest season since 1931 Rainy season
	Fall	17.97	486.88	3rd hottest season since 1931 3rd wettest season since 1931
2007	Winter	9.5	215	Very dry season
	Spring	14.2	133.64	Driest March of the XXI century
	Summer	21	86.36	Lowest value of air mean temperature (mean) since 1990 Wettest season of the XXI century
	Fall	16.75	120.92	Driest season of the XXI century and the 6th driest since 1931 Driest October of the XXI century
2008	Winter	10.39	196.03	4th highest air maximum temperature since 1931 Very dry season January: 2nd highest value of the maximum temperature since 1931
	Spring	13.69	252.41	15th consecutive value above the normal air mean temperature (mean) of the 1971-2000 period Wettest season since 2001
	Summer	19.23	25	Dry season
	Fall	15.43	109.31	September: highest value of total precipitation recorded since 1965 (147.3 mm) November: persistence of very low air minimum temperatures
2009	Winter	10.13	288.45	Cold days and nights
	Spring	15.1	96.3	16th consecutive value above the normal air mean temperature (mean) of the 1971-2000 period March and May: air maximum temperatures much higher than the 1971-2000 mean values Driest season since 1931
	Summer	20.2	61.84	Three heat waves
	Fall	17.87	222.35	6th hottest season since 1931 (mean and minimum temperatures) 8th hottest season since 1931 (maximum temperatures) Two heat waves

[1]Approximated values.

Considering the four studied regions in the 2006-2009 period, absolute frequencies of each species belonging to Obsoletus group, as well as minimum and maximum mean temperatures, are represented in Figure 3.9.

Figure 3.9. – Distribution of Obsoletus group species in the four regions of mainland Portugal (2006-2009).

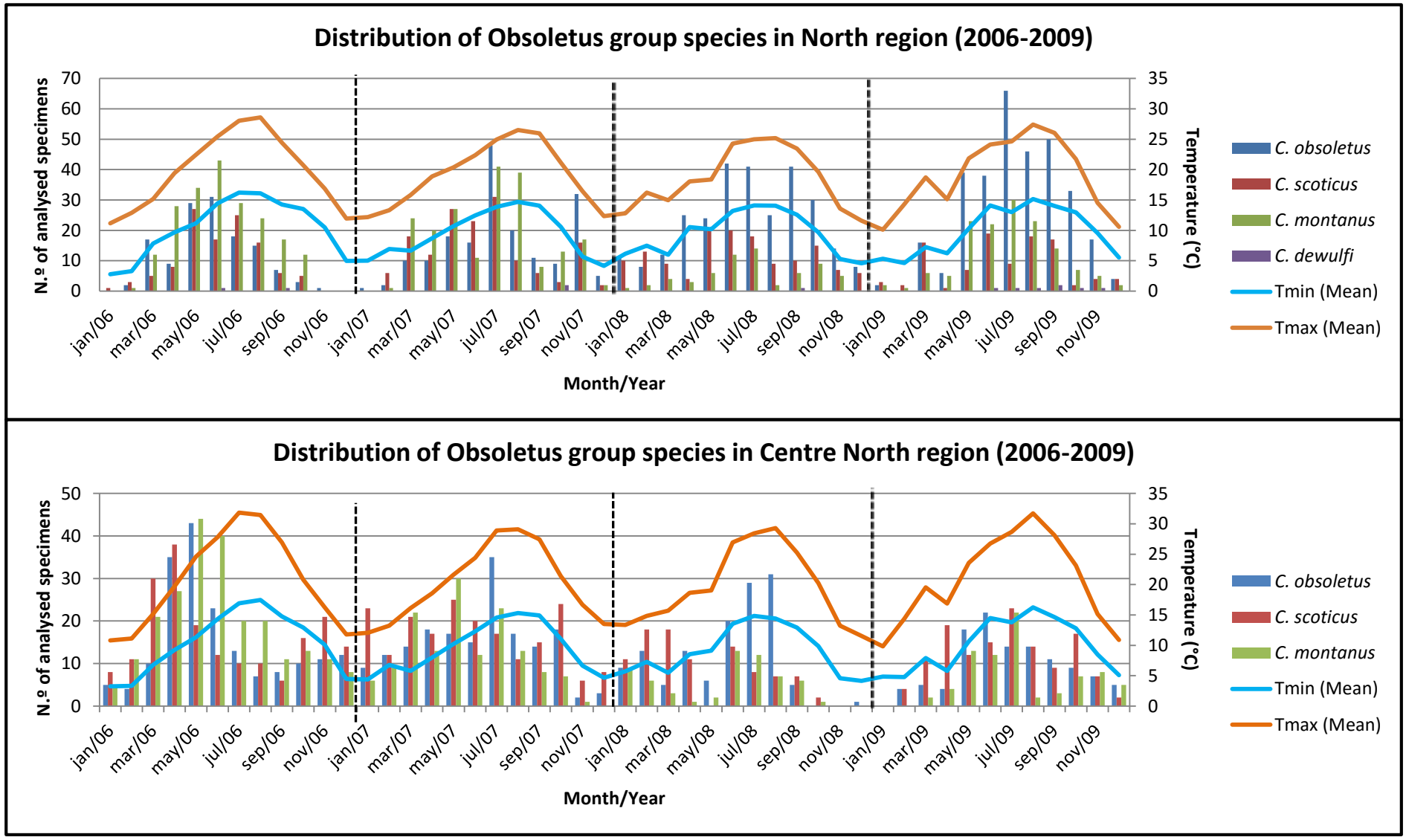
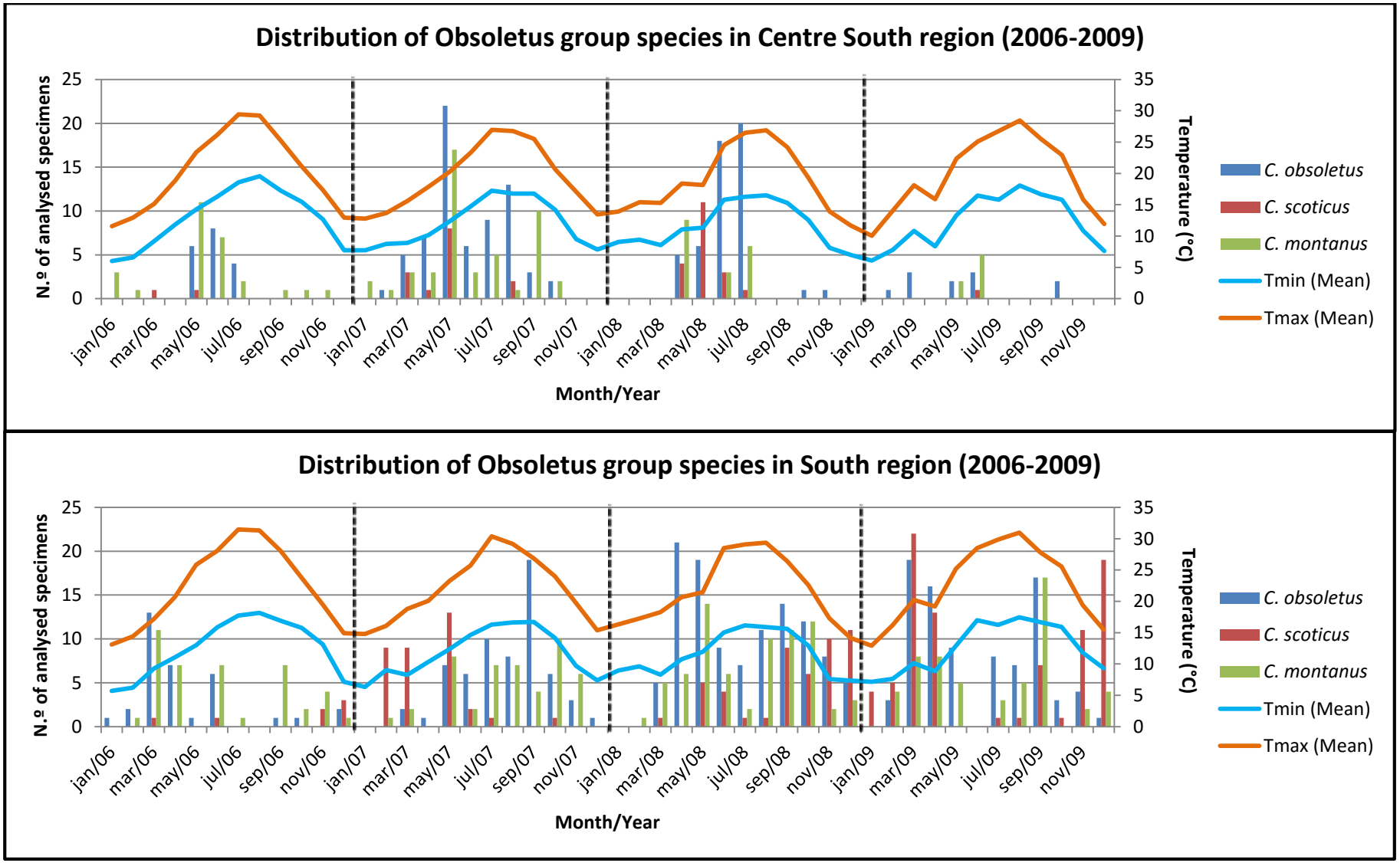


Figure 3.9. – Distribution of Obsoletus group species in the four regions of mainland Portugal (2006-2009) (Continuation).



The occurrence of Obsoletus group species per season is shown in Table 3.8. Relative frequencies were normalized with the maximum specimens analysed per capture (8 females).

Table 3.8. – Relative frequency of Obsoletus group species captured per season and per year in each region of mainland Portugal.

Season	Year	Region of Mainland Portugal (%)[1]			
		North	Centre North	Centre South	South
Winter	2006	21.88	25	6.25	5.56
	2007	31.25	69.08	12.50	15.38
	2008	51.92	41.85	0	2.78
	2009	37.50	28.13	4.17	36.46
Spring	2006	70.42	87.83	39.58	41.67
	2007	57.64	79.02	34.50	29.17
	2008	43.15	22.35	31.25	36.54
	2009	46.48	39.29	4.38	40.32
Summer	2006	59.51	58.71	26.25	26.79
	2007	82.99	84.90	22.16	44.79
	2008	55.79	40.99	37.50	28.98
	2009	72.87	55.65	7.03	18.38
Fall	2006	46.43	44.58	2.88	15.18
	2007	54.17	38.31	10.12	24.50
	2008	66.35	13.13	1.79	33.87
	2009	63.75	31.45	1.67	35.23

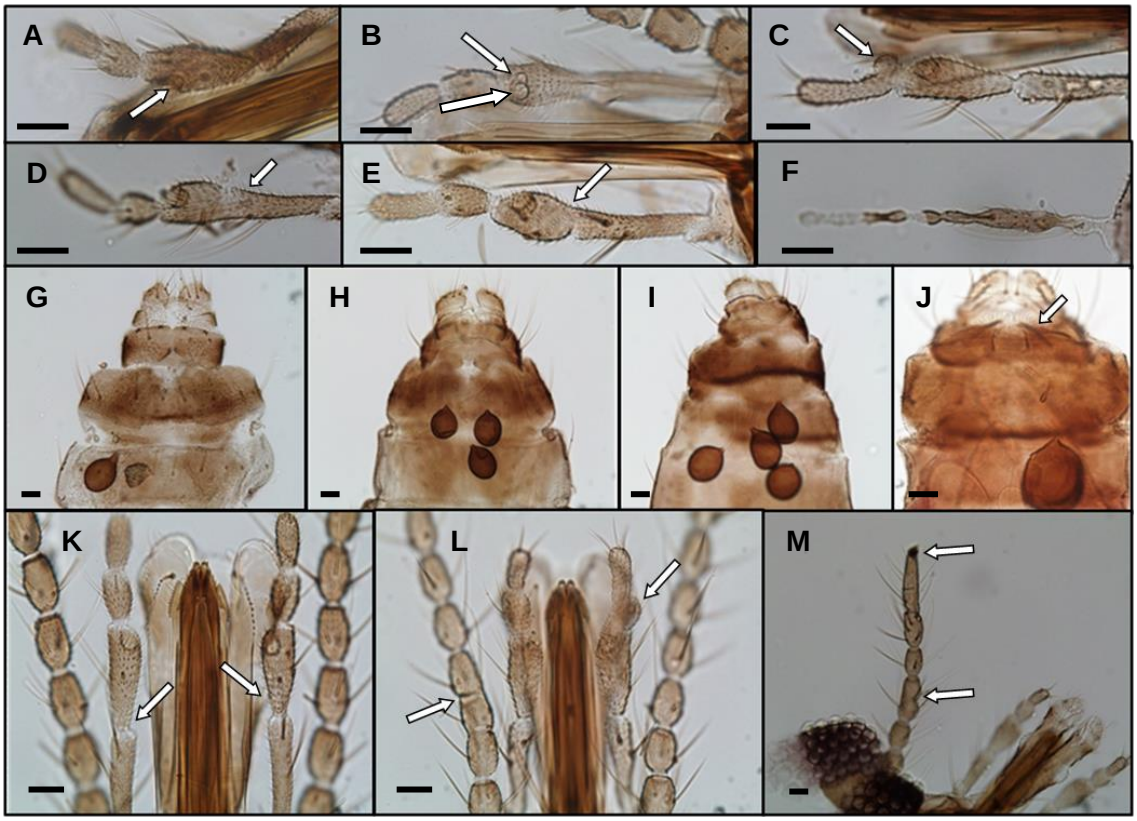
[1]Relative frequencies were normalized considering the maximum specimens analysed per capture (8 females).

3.4.4. Anatomical aberrations

Some female midges possessed aberrant anatomical aspects, like defective or doubled sensorial pits and unequal lengths on the 3rd palpus segment, palpi and antennae with defective or fused articles, genitalia with male characteristics and presence of 1, 3 and 4 functional spermathecae (Figure 3.10.).

Furthermore, it was observed one specimen whose spermathecae had perforations and four specimens with nematode parasites inside their abdomen. None of these parasitized individuals had genital modifications or were intersexual individuals.

Figure 3.10. – Anatomical aberrations observed in Obsoletus group specimens.



A – Defective sensorial pit; B – Double sensorial pit; C – Fused palpus segments; D, E and F – Defective palpus segments; G, H and I – Irregular number of functional spermathecae (one, three and four respectively); J – Specimen with male genitalia and spermathecae; K – Unequal 3rd palpus segment length; L – Fused antennal flagellomeres and palpus segments; M – Defective antennal flagellomeres. Scale bars: 20 µm. Original photos.

The distribution of morphological aberrations between different Obsoletus group species and *Culicoides* spp. (n=156; 3.1%) is shown in Tables 3.9. and 3.10..

Table 3.9. – Distribution of anatomical aberrations found in species from Obsoletus group.

Anatomical aberrations		***Culicoides* species**			**Total**
		C. obsoletus	***C. scoticus***	***C. montanus***	
3rd palpus segment	Defective sensorial pit	3	1	5	9
	Double sensorial pit	7	0	3	10
	Unequal length	9	11	15	35
Palpus	Defective articles	12	4	8	24
	Fused articles	8	2	4	14
Antenna	Defective articles	1	0	2	3
	Fused articles	1	1	1	3
Genitalia	With male characteristics	5	2	3	10

Table 3.9. – Distribution of anatomical aberrations found in species from Obsoletus group (Continuation).

Anatomical aberrations		***Culicoides* species**				**Total**
		C. obsoletus	***C. scoticus***	***C. montanus***	***C. dewulfi***	
Number of spermathecae	One	2	0	2	0	4
	Three	19	4	6	1	30

Table 3.10. - Distribution of anatomical aberrations found in *Culicoides* spp. and proposed species distribution.

Anatomical aberrations		***Culicoides* spp.**	**Proposed species distribution[1]**
3rd palpus segment	Defective sensorial pit	5	*C. obsoletus*: 1 *C. scoticus*: 3 *C. montanus*: 1
	Double sensorial pit	3	*C. obsoletus*: 1 *C. scoticus*: 2
	Unequal length	1	*C. obsoletus*
Palpus	Defective articles	1	*C. obsoletus*
	Fused articles	2	*C. obsoletus*
Genitalia	With male characteristics	1	*C. obsoletus*
Number of spermathecae	Four	1	*C. scoticus*

[1]Proposed species were based exclusively in spermathecae length.

3.4.5. Anatomical measures and other observations

The values obtained for *C. montanus* specimens (n=502) concerning the 3rd palpus segment ratio L/W and spermathecae length are presented in Table 3.11.

Table 3.11. – 3rd palpus segment L/W ratio and spermathecae length for *C. montanus*.

3rd palpus segment L/W ratio						**Spermathecae length (µm)**		
Minimum	**Q_1**	**Median**	**Q_3**	**Maximum**	**Mean**	**Minimum**	**Mean**	**Maximum**
1.66	2.32	2.465	2.58	3.14	2.4491	37.5	50.36	62.5

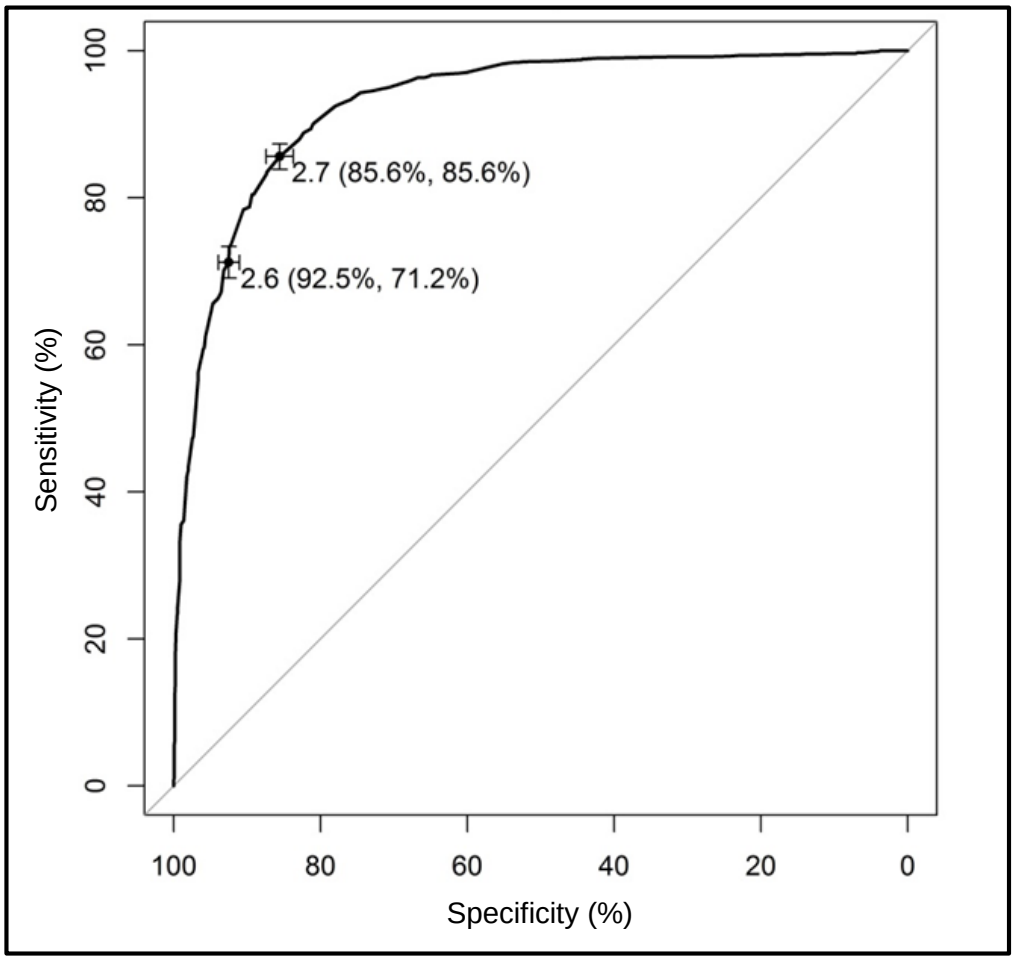
Chitinous plates conformation was registered for both *C. obsoletus* (n=938) and *C. scoticus* (n=624) species (Table 3.12.).

Table 3.12. – Chitinous plates conformation for *C. obsoletus* and *C. scoticus* species.

***Culicoides* species**	**Chitinous plates shape**		**Total**
	Parallel	**Convergent**	
C. obsoletus	748	190	938
C. scoticus	391	233	624
Total	1139	423	1562

With the ratios L/W of the 3rd palpus segment obtained from *C. obsoletus* and *C. scoticus* specimens (n=2931), a ROC was constructed and a more accurate cut-off value for both species were determined (2.7), as well as its sensitivity (85.6%) and specificity (85.6%) (Figure 3.11.). The sensitivity and specificity of 2.6 cut-off value were also determined (92.5% and 71.2%, respectively) for comparison with those of 2.7 cut-off value (Figure 3.11.).

Figure 3.11. – Cut-off values of the 3rd palpus segment length/width ratio.



Specificity and sensitivity for each cut-off value is represented within brackets.

3.5. Discussion

Species within Obsoletus complex (Meiswinkel *et al*., 2004a; Garros *et al*., 2010; Harrup *et al*., 2014) both have very similar phenotypical and molecular characteristics between them:

1) The only evident anatomic difference between *C. montanus* and *C. obsoletus* females is the depth of the 3rd palpus segment sensorial pit (Shakizjanova, 1962; Gutsevich, 1966; Gutsevich, 1973; Mirzaeva, 1984; Glukhova, 1989; Mirzaeva, 1989; Mathieu *et al*., 2012; Kirkby & Dominiak, 2014).

2) *C. obsoletus* and *C. scoticus* share a similar ITS-1 sequence, with 93.3% and 95.5% of concordance (Nielsen & Kristensen, 2011).

The undiagnosed species found in this work have anatomical combinations of the three recognised species inside Obsoletus complex, like specimens of *Avaritia* subgenus observed elsewhere (Gomulski *et al*., 2005; Sanders *et al*., 2012; Wenk *et al*., 2012; Harrup *et al*., 2013; Meiswinkel *et al*., 2014b). Due to their high similarity, they are probably not yet genetically separated from parent species, being hybrid specimens with mixed anatomical features of recognised species. The high number of specimens present in some of the selected farms (1, 2 and 6) shows that, maybe, mating between closed related species is occurring in these regions. Very likely, these specimens will follow a genetically separated line and form isolated species in a near future. Molecular identification of these specimens should be conducted in order to understand their taxonomic position, in an integrative taxonomy approach (Harrup *et al*., 2014).

Concerning specimens with one spermathecae <57 µm and another >59 µm they all have anatomic features similar to *C. obsoletus* species. In this way, the present work suggests that spermathecae interval defined for this species by Delécolle (1985) should be redefined from [46-59] µm to [37.5-62.5] µm, being the same interval determined for *C. montanus* in this study. *C. scoticus* interval concerning spermathecae length should also be redefined from [57-67] µm to [57-95] µm. *C. dewulfi* should have their spermathecae length intervals also redefined from [40-44] µm to [40-46.25] µm for the smallest and from [55-59] µm to [53.75-59] µm for the biggest.

From this study it can be observed that species within Obsoletus group are unequally distributed in mainland Portugal. The mean values from the 2006-2009 period show that they are predominantly present in the North and Centre North regions of mainland Portugal (70.01%), being less common in Centre South (10.57%) and South (19.42%) regions. These values are in agreement with other authors (Capela *et al*., 2003; Pena, 2003; Brugger & Rubel, 2013; Ribeiro *et al*., 2015), showing a preference of these species for edaphoclimatic conditions of the northern zones of mainland Portugal. The raise observed in South region, when compared with Centre South region, may be due to a less hot and dry climate observed in the former during the summer (IPMA, 2015). This contributed for a new occurrence of Obsoletus group midges in fall, situation that did not occur in Centre South region in 2008 and 2009 (Figure 3.9., page 123).

A single male *C. chiopterus* was caught in 1979 in Aldeia Nova (North region of mainland Portugal, near the Spanish border) using a modified New Jersey trap (Capela *et al*., 1992; Pena, 2003). If *C. chiopterus* midges are present in our country, their numbers are very low or CDC light traps probably could not be efficient enough for catching midges from this species. *C. dewulfi* was referred for the first time in our country, but it was only found on three farms from the North region of mainland Portugal and in small numbers (n=12 in the 2006-2009 period), probably because of the same reasons pointed out for *C. chiopterus*.

Gomulski *et al*. (2005) referred that, although *C. dewulfi* may also be responsible of BTV transmission in Italy, the relative abundances of *C. dewulfi* are low and it probably does not play a pivotal role on that transmission. Furthermore, Venail *et al*. (2012) also observed that, during 2002-2008 period, both *C. dewulfi* and *C. chiopterus* species increase their abundance from Southern to Northern areas of France, reaching only six individuals of the former and no specimens of the second in Corsica island (which is localized nearly at the same latitudes of mainland Portugal regions where *C. dewulfi* was found), while in Northern regions *C. dewulfi* reached up to 20% and *C. chiopterus* 35% of the total annual catches. This is in agreement with a better tolerance of *C. chiopterus* to colder regions than *C. dewulfi* species (Sprygin *et al*., 2014; Steinke *et al*., 2015). *C. dewulfi* is ocasionally found in all Northern regions of Spanish territory, with a low abundance, while *C. chiopterus* is a rare species in Spain and Basque Country (De Heredia & Lafuente, 2011).

Concerning Obsoletus complex, all three species present in Western Europe were identified in this study. Thus, *C. montanus* was also referred for the first time in Portugal, raising the number of identified *Culicoides* species to 66.

Pena (2003) referred the occurrence of *C. obsoletus* mainly in Northern regions of mainland Portugal, being dispersed by all territory, with the exception of Centre South region. *C. scoticus*, although present, was rarer and more dispersed in Portuguese territory.

This work also shows that Obsoletus complex species are not equally distributed between them in different regions of mainland Portugal. *C. obsoletus* is the most frequent species in all regions of mainland Portugal, with exception of Centre North, where numbers almost equal those of *C. scoticus*, the most common species in this region (*C. obsoletus:* 34.56%; *C. scoticus*: 35.89%). *C. montanus* is the second most common species within Obsoletus complex, with the exception of Centre North region, where it is the third, with 29.55%. When the distribution of different species is analysed, it can be observed that *C. obsoletus* species is equally distributed in all four studied regions of mainland Portugal, with a minimum (22.09%) and a maximum (26.66%) of relative frequencies in Centre North and South regions, respectively. However, *C. scoticus* is concentrated in northern regions of mainland Portugal, reaching a total of 75.30%, but only 6.08% in Centre South region and 18.62% in South region. *C. montanus* distribution rises from North to South of mainland Portugal, being almost equal in North and Centre North regions (mean of 21.08%) and reaching 31.76% in South region.

These results show differences with those observed previously by Pena (2003), since *C. scoticus* was referred as being a scarce species and *C. montanus* was not identified at all. The major reason may rely on more accurate methods of identification available nowadays (*e.g.*, Mathieu *et al*., 2012), which allow the identification of very similar species (*C. obsoletus* and *C. montanus*) (Mathieu, 2011; Mathieu *et al*., 2012; Kirkeby & Dominiak, 2014) and understanding their real distribution in mainland Portugal.

Furthermore, and comparing with other authors, de Heredia & Lafuente (2011) reported that *C. obsoletus* is abundant and is largely widespread in Spain, especially in the Northern regions, while *C. scoticus* is less frequent but also well spread throughout all Spanish territory. Baldet, Delécolle, Mathieu, de La Rocque & Roger (2004) observed that *C. scoticus* were present in larger numbers than *C. obsoletus* in Corsega island. Also, Pili, Carcangiu, Oppo & Marchi (2010) observed a higher proportion of *C. scoticus* than *C. obsoletus*, particularly in the first 3 to 4 months of the year, in Sardinia island. These findings suggest that *C. scoticus* could be a good candidate for the transmission and maintenance of BTV in late winter to early spring in that region. Carpenter *et al*. (2008b) demonstrated in experimental infections using BTV-8 and BTV-9 that *C. scoticus* was infected with a higher virus titer than *C. obsoletus*. Furthermore, Elbers *et al*. (2013) confirmed that, in field caught Obsoletus complex, the rate of *C. scoticus* SBV positive females was higher than SBV positive *C. obsoletus* females. However, in a study performed in Belgium by De Regge *et al*. (2012), SBV was not detected in *C. scoticus* but only in *C. obsoletus*. In contrast, in Italy, *C. obsoletus* resulted as the most abundant species of the Obsoletus complex in the area where the SBV circulated, being positive to SBV (Sarvašová *et al*., 2014).

Since the vector competence for virus transmission inside Obsoletus complex is not identical (Sarvašová *et al*., 2014), this unequal distribution of Obsoletus complex species must be taken into account when referring to arboviruses transmission (like BTV or SBV) in our country. This different geographical distribution of species within Obsoletus complex may be due to different ecology preferences, different animal species in the surrounding of the traps or to different edaphoclimatic conditions (Sarvašová *et al*., 2014) of mainland Portugal.

According to Venail *et al*., (2012), Corsica island presents a Mediterranean climate characterized by dry and hot summers and wet and mild winters, which is the same observed in regions of mainland Portugal below approximately 39.66025 N latitude and Beira Baixa region (Peel *et al*., 2007; Brugger & Rubel, 2013). In this island, *C. obsoletus*/*C. scoticus* populations seem to be limited by dryness. Both species were abundant around May and declined during summer dry months, while in rainy summers population could increase again. Besides, both species became usually rare in January except in years when populations were present for 12 months.

Briefly, this work showed that midges belonging to Obsoletus group can have multiple generations per year, probably due to Portuguese edaphoclimatic conditions and, sometimes, they can be present throughout the year. However, very hot and dry weather or heavy rainfall conditions have a negative impact in the abundance and occurrence of these midges (Venail *et al*., 2012). The year of 2009 is a good example to show how climate negatively influences Obsoletus group species occurrence: a spring with a dry and hot weather, a summer with three hot waves and an extremely dry fall in Centre South region of mainland Portugal resulted in a collection of only 3.13% of specimens belonging to this

group. In addition, moderate rainfall together with reduction of dryness and mild temperatures favours the occurrence of Obsoletus group midges. All species from Obsoletus complex are well adapted to the mainland Portugal climate, with the exception of Centre South region, mainly ascribed to the dryness of this Portuguese territory, especially during summer.

To the best of our knowledge this work refers for the first time the following morphological aberrations in *Culicoides* specimens: defective sensorial pits and unequal length of the 3rd palpus segment, palpus and antenna with defective articles and specimens with one and four spermathecae. The perforation of spermathecae observed in one specimen may be an artefact of preparation. The unequal length of 3rd palpus segment and the presence of three functional spermathecae instead of two were the most common anatomical alterations observed in *Culicoides* midges from Obsoletus group.

These alterations observed in *Culicoides* midges should be analysed with caution, since their presence can be responsible for unaccurate species classification (de Heredia & Lafuente, 2011). Since each female can deposit 10 to 675 eggs according to species, there is a huge probability for anatomical aberrations to occur due to genetic or morphogenetic malformation (Felippe-Bauer & Silva, 2006; de Heredia & Lafuente, 2011). These modifications appear in laboratorial preparations because they are compatible with life and maintained in adult specimens. *Culicoides* with anatomical changes which are not compatible to life do not reach the adult phase or do not live long enough to be captured with light traps, being this condition in agreement with Darwin's theory (Darwin, 1859).

In Nielsen and Kristensen (2011) work, no *C. montanus* specimens were observed, since this species is only present in Western Europe region. With this study, the intervals concerning 3rd palpus segment L/W ratio, as well as spermathecae length, were defined for this species, showing that values overlap those registered for *C. obsoletus* species, since they are very close related species (Mathieu *et al*., 2012; Kirkby & Dominiak, 2014)

With a bigger number of analysed specimens than those observed by Nielsen & Kristensen (2011) (20 against 2931), a better approach to *C. obsoletus* and *C. scoticus* identification using the 3rd palpus segment L/W ratio was performed. The new results obtained demonstrated that specimens with the L/W ratio <2.7 should classified as *C. obsoletus* and as *C. scoticus* when ratio L/W is above this same value, giving a sensitivity and a specificity of 85.6% to this diagnostic test, raising the sensitivity in 14.4% but lowering the specificity in 6.9% when comparing the cut-off value of the previous test (2.6, with 92.5% and 71.2% of sensitivity and specificity, respectively).

Concerning chitinous plates conformation, this feature should not be used to identify these two species, since both conformations are observed in *C. obsoletus* and *C. scoticus*, not being a helpful diagnostic tool for specimens identification, as it was also observed by other authors (Pages & Sarto I Monteys, 2005).

Avaritia subgenus includes a large proportion of *Culicoides* midges implicated in the transmission of several arboviruses to animals, especially the species belonging to Obsoletus group. Considering that these species do not have an identical vector competence, it is extremely important to know their distribution in a region or area. However, since females have the same wing pattern and are very difficult to distinguish between them in laboratory routine, Obsoletus group is mainly reported as so in all European countries during surveillance programs. Furthermore, there is very little information concerning edaphoclimatic conditions which influence positively or negatively these midges occurrence.

The presence of 'isomorphic' species, i.e. genetically distinct, morphologically indistinguishable 'sibling' species which frequently occur sympatrically (like Obsoletus group species), are also difficult to identify on the basis of morphological characteristics and sometimes requires the use of molecular methods for separation. Regarding vector-borne diseases, failure to recognise cryptic species may confound epidemiological investigations due to potential variation in vector competence and/or host preference between sibling species and may complicate control efforts due to variation in the bionomics of sibling species (Harrup *et al*., 2014).

Thus, this work contributed for a refinement of existing diagnostic methods concerning morphological identification of species belonging to Obsoletus group, namely 3rd palpus segment L/W ratio, as well as spermathecae size, which are extremely useful anatomic features to define species with a high level of certainty for Obsoletus group and Obsoletus complex. The existence of intermediary forms in this complex increases the difficulty concerning species identification, since they can be a result of intraspecific variation, hybrids or even new species. Further morphological and molecular investigation within this complex is needed to understand the taxonomic position of these specimens. The report of different types of anatomical aberrations is also important, since these morphological alterations can be misleading during specimen's identification. Finally, an exhaustive work concerning *Culicoides* midges identification inside Obsoletus complex, together with climate analysis in different seasons, give a better knowledge about the different species preferences for Portuguese climate, as well as their distribution in Portuguese territory.

Chapter 4: Ecologic characterization of *Culicoides* species in mainland Portugal

4.1. Introduction

VBD are exquisitely sensitive to environmental change because of complex ecological processes that regulate the distribution and abundance of vectors in the environment, their contact with humans and often also non-human reservoir hosts of infection for vectors (Sutherst, 2004). No other infectious disease threats of humans exhibit such extensive dependence upon ecological complexity. Historically, successful VBD prevention has relied upon the management or elimination of vector populations within the environment (Lemon *et al*., 2008). To understand the dynamic of VBD is crucial to recognize the factors that influence all of its components, like the interactions between vectors and their physical or biological environment.

New technologies that have tremendous potential for improving our understanding of relationships between the environment and VBD include remote sensing by Earth-orbiting satellites, geographic information systems and spatial statistics (Fish, 1996). A group of satellites continuously acquires a broad range of environmental data on vegetation, water, atmosphere, land use, and weather on a global scale that are achieved and available for research and applications in VBD (Beck, Lobitz & Wood, 2000).

Outbreaks of severe BTD can be economically devastating but, because BTV is transmitted by incompletely defined species of a relatively ubiquitous but poorly characterized genus of insect vector (Mellor *et al*., 2000; Carpenter *et al*., 2008c), elimination of the infection in enzootic areas is difficult or impossible. *Culicoides* biting midges occur throughout most inhabited world where they transmit a wide variety (>50) of pathogens of animals and humans, including not only orbiviruses such as BTV, AHSV and EHDV, but also rhabdoviruses, reoviruses and pathogenic bunyaviruses such as Akabane and Schmallenberg viruses (Mellor *et al*., 2000; Conraths, Peters & Beer, 2013).

Of relevance to the possible future emergence of new zoonotic diseases, some *Culicoides* midges have a broad host feeding preferences that include humans as well as animals, and animal parasites can be transmitted to humans through the bites of *Culicoides* midges (Calvo *et al*., 2012; Lassen *et al*., 2012a; Santiago-Alarcon, Havelka, Schaefer & Segelbacher, 2012).

Concerning the abovementioned diseases, an evaluation of geographical areas where major vectors are present is essential, as well as the identification of “non-vectors” species that are present in regions where outbreaks occur, since they can be also involved in that transmission (Mellor *et al*., 2000; Wilson & Mellor, 2008). Negligence of these factors could result in serious problems to the economy of affected countries, since unexpected outbreaks of diseases transmitted by these midges can occur, like it happened in the past in European continent (Carpenter *et al*., 2009; Wilson & Mellor, 2009; Ganter, 2014). As example, BTD results in annual losses of approximately $3 billion due to morbidity and mortality of affected animals, trade embargoes and vaccination costs (Osburn, 2008; Maclachlan & Mayo, 2013).

A lot of data about environment preferences and ecology is lacking for *Culicoides* biting midges, in particular their distribution, soil moisture and larval ecology and breeding or resting sites (Mellor *et al*., 2000; Lühken *et al*., 2014; Zimmer *et al*., 2013a; Zimmer *et al*., 2015). Furthermore, more controlled experiments concerning environment (*e.g.*, soil characters, type of vegetation) and management factors (*e.g.*, manure storage) are needed to understand the ecological processes that affect *Culicoides* distribution in and around farms, including for main vectors such as *C. imicola* (Mellor *et al*., 2000; Peters *et al*., 2014; Scolamacchia *et al*., 2014). Relative host preference rate is one of the most important parameters used for disease transmission modelling (Hartemink *et al*., 2009; Szmaragd *et al*., 2009) but it remains a neglected area of investigation (Lo Iacono *et al*., 2013).

Thus, since not all *Culicoides* species have the same edaphoclimatic preferences and concerning the lack of knowledge about the ecology of these midges, an accurate study should be performed in order to understand *Culicoides* population dynamics, their interaction with the environment and their role in VBD transmission.

4.2. Objectives

The main objectives of this work were:

1) Evaluation of presence and absence of *Culicoides* species, mainly those recognised as or potential vectors of BTV, SBV and AHSV, per season, using edaphoclimatic information available in databases.
2) Evaluation of the occurrence and dispersal risk of the abovementioned *Culicoides* species during the year in mainland Portugal.
3) Mention of the meteorological conditions that took place during the remaining *Culicoides* species occurrence as complementary information.

4.3. Material and methods

4.3.1. NESP for BTD and insect sampling

C. imicola, *C. pulicaris*, species from Obsoletus group (recognised vectors of BTV, SBV and AHSV), *C. newsteadi* and *C. punctatus* (potential vectors of BTV) were captured in the scope of the NESP for BTD and analysed as described in Chapter 2 (see 2.3.1., 2.3.2. and 2.3.3.). The time division considered was the season of the year, as described in Chapter 3 (see 3.3.2.) and a separated analysis was performed for each season. For the 2005-2013 period and concerning each season (spring, summer and fall) a total of 120 farms from mainland Portugal were selected for this study, while for winter only 80 farms were selected, due to less captures performed during this season after Official Veterinary services decision. In each selected farm and for each season, the presence/absence of the abovementioned *Culicoides* species during the 2005-2013 period was registered.

4.3.2. Edaphoclimatic information and statistical analysis

For *C. imicola*, *C. pulicaris*, species belonging to Obsoletus group, *C. newsteadi* and *C. punctatus* species, edaphoclimatic information were obtained from Bioclim (WorldClim – Global Climate Data, n.d.) (19 variables) and CORINE Land Cover (European Environment Agency, 1995) (44 variables) databases. CORINE Land Cover layers were processed using QGIS 2.10.1 to produce maps with the minimum distance to each of the land cover classes. Together with information obtained from presence (captured specimens) and absence (no captured specimens) of referred *Culicoides* species in different seasons in mainland Portugal, a statistical analyses using R Studio® software was performed with the following steps:

1) The collinearity between the 63 variables was evaluated and the variables with less biologic impact and higher Pearson correlation coefficient ($|r| > 0.7$) were excluded to avoid collinearity in the final model. Table 4.1. shows the variables chosen for this work. Due to the high collinearity between climate variables, only two of them (mean temperature of the wettest quarter and mean temperature of the driest quarter) were selected to be included in the model.

Table 4.1. – Climate and land cover variables chosen for model analysis.

Variable Type	Variable code	Mean
Climate (n=2)	Bio8	Mean temperature of wettest quarter
	Bio9	Mean temperature of driest quarter
Land Cover (n=22)	111	Continuous urban fabric
	112	Discontinuous urban fabric
	132	Dump sites
	133	Construction sites
	141	Green urban areas
	211	Non-irrigated arable land
	212	Permanently irrigated land
	222	Fruit trees and berry plantations
	223	Olive groves
	231	Pastures
	244	Agro-forestry areas
	311	Broad-leaved forest
	312	Coniferous forest
	313	Mixed forest
	321	Natural grassland
	322	Moors and heathland
	411	Inland marshes
	412	Peatbogs
	421	Salt marshes
	511	Water courses
	512	Water bodies
	523	Sea and ocean

2) For each species and each season, an univariate analysis was performed taking into account 75% of the records and those that were statistically significant (which had a *p*-value below a pre-defined threshold of p <0.1) with the response variable (probability for that species to occur in that season and region) were selected.

3) A multivariable logistic regression model was built, where those variables that did not improve the final model, i.e. with a higher AIC value, were excluded, using a backward-forward variable selection procedure, and the regression model was obtained. The regression model was based in the following mathematical expression: $\mathrm{Y} = \log\left(\frac{P}{A}\right) = \alpha + + \beta i \mathcal{X} i$, where Y is the response variable (probability of a species to be present in a determined area), P is the species presence, A is the species absence, α is a coefficient representing the intersection value with Y axis when X is zero, $\mathcal{X} i$ is the variable (climate or land cover) value in one specific point and βi the coefficient of that respective variable defined by the program when the model is finished; it must be referred that, concerning land cover, a positive value corresponds to a minor risk when the variable is closer to the capture point and a negative value means the opposite; concerning climate, a positive value means that higher temperatures favour midges occurrence while a negative value means the opposite.

4) Finally, model validation was performed with the remaining 25% of the collecting points (those that were not included in the first analysis) and a cut-off point was defined to consider when a *Culicoides* biting midge is effectively present or absent, with a respective level of sensitivity and specificity

5) After the model was obtained, probability maps concerning species presence or absence were elaborated.

For the remaining species (with exception of *C. fagineus*, *C. kingi* and *C. paolae*, since only one specimen of each species was identified), temperature, average wind speed and relative humidity, were obtained from the closest meteorological station to the trapping site. Humidity was recorded at 3 pm on the day the trap was placed and 9 am on the day of collection to reflect conditions corresponding to the evening/night of collection.

4.4. Results

4.4.1. Edaphoclimatic variables influence

The variables which positively or negatively influence the occurrence of the referred *Culicoides* species per season are represented in Table 4.2.

Table 4.2. - Variables which influence positively and negatively the occurrence of different *Culicoides* species per season (2005-2013).

***Culicoides* Species**	**Season**	**Variables**	**β value**	**α value**
C. imicola	Spring	Bio8 Bio9 211	0.0301 0.0320 -0.0002	-9.2993
	Summer	Bio9 212	0.1032 -9.561×10^{-5}	-21.72
	Fall	Bio8 Bio9 133 211 222 223 322	0.0966 0.1726 1.141×10^{-4} -2.487×10^{-4} -7.809×10^{-5} 4.719×10^{-5} -5.067×10^{-5}	-45.90
	Winter	Bio9 112 512	0.0601 2.595×10^{-4} -1.416×10^{-4}	-13.86
Obsoletus group	Spring	211 244	3.951×10^{-4} 1.062×10^{-4}	0.6866
	Summer	244 321	3.356×10^{-5} -4.760×10^{-5}	1.136
	Fall	Bio8 Bio9	-0.0192 -0.0605	15.4369
	Winter	231 244 512	4.861×10^{-5} 2.875×10^{-5} 7.156×10^{-5}	-1.080
C. pulicaris	Spring	Bio9 132 313 321	-0.0276 -1.438×10^{-5} -1.055×10^{-4} -8.173×10^{-5}	6.940
	Summer	231 244 313	-7.218×10^{-5} 2.581×10^{-5} -1.387×10^{-4}	0.3997
	Fall	222 311 313	4.069×10^{-5} 2.314×10^{-4} -1.328×10^{-4}	-1.497
	Winter	Bio9 311	-0.0599 0.0002	10.7438
C. punctatus	Spring	321	-6.126×10^{-5}	2.638
	Summer	411	1.058×10^{-5}	0.6107
	Fall	133	2.310×10^{-5}	0.3572
	Winter	133	3.5×10^{-5}	0.2163
C. newsteadi	Spring	Bio8 244	0.0367 -3.302×10^{-5}	-2.225
	Summer	244 321 511	-3.961×10^{-5} 1.097×10^{-4} -3.509×10^{-5}	1.141

Table 4.2. - Variables which influence positively and negatively the occurrence of different *Culicoides* species per season (2005-2013) (Continuation).

***Culicoides* Species**	**Season**	**Variables**	**β value**	**α value**
C. newsteadi	Fall	Bio8	0.042	-0.7937
		112	-2.443×10^{-4}	
		223	-4.442×10^{-5}	
		244	-3.898×10^{-5}	
		511	-2.597×10^{-5}	
	Winter	Bio8	0.0459	-4.553
		223	-4.256×10^{-5}	
		312	1.413×10^{-4}	

C. imicola presence is favoured by high temperatures during the driest quarter in all seasons. In spring and fall seasons, higher temperatures during the wettest quarter also favour the occurrence of this species. Concerning edaphic variables, non-irrigated arable land is favourable for *C. imicola* occurrence during spring, while permanently irrigated land is favourable during summer. More variables affect this species during fall, being non-irrigated arable land, fruit trees, berry plantations, moors and heathland favourable variables and construction sites together with olive groves non-favourable ones for its occurrence. During winter, *C. imicola* tend to appear near water bodies, while discontinuous urban fabric terrain does not favour its appearance.

Concerning Obsoletus group species, the models show that agro-forestry areas do not favour their occurrence near capture points during spring, summer and winter seasons. In spring, non-irrigated arable land does not favour these species occurrence. In summer, natural grassland raises the risk for these species appearance. In fall, the mean temperatures of the wettest and the driest quarters should be low for Obsoletus group species to occur. Finally, in winter, both pastures and water bodies do not favour their occurrence.

Mixed forest nearby the capture point raise the risk of *C. pulicaris* occurrence in spring, summer and fall seasons, while broad-leaved forest do not favour the appearance of this species in fall and winter seasons. Concerning the mean temperature of the driest quarter, lower temperatures favour this species occurrence during spring and winter seasons. In spring, dump sites and natural grassland raise the risk of this species appearance in the capture points. In summer, pastures favour their occurrence while agro-forestry areas do not. Finally, during fall, fruit trees and berry plantations near the capture point diminishes the chance of *C. pulicaris* occurrence.

The presence of natural grassland near the capture point favours the occurrence of *C. punctatus* species during spring. In summer, inland marshes do not favour this species appearance, as well as construction sites, which diminishes the risk of this species presence nearby the capture point during fall and winter seasons.

C. newsteadi is positively influenced by higher mean temperatures during the wettest quarter of spring, fall and winter seasons. Agro-forestry areas also raise the probability of *C. newsteadi* appearance in spring, summer and fall seasons, as well as olive groves favour their presence in fall and winter seasons. Water courses raise the probability of *C. newsteadi* appearance during summer and fall seasons. Natural grasslands and coniferous forests do not favour its occurrence in summer and winter seasons, respectively. Also during fall, discontinuous urban fabric terrain favours the occurrence of this species in farms.

For the remaining species referred in mainland Portugal the meteorological data obtained from the closest meteorological station to the farms are expressed in Annex 4.1. as complementary information.

4.4.2. Cut-off points and presence/absence probability maps

The cut-off points, with the respective sensitivity and specificity mean values for each species and per season, are represented in Table 4.3..

Table 4.3. – Cut-off points with the correspondent sensitivity and specificity mean values for each species and per season.

***Culicoides* Species**	**Season**	**Cut-off point**	**Sensitivity (%)**	**Specificity (%)**
C. imicola	Spring	0.6592	80	82.86
	Summer	0.6254	89.29	81.25
	Fall	0.6864	95	100
	Winter	0.5959	57.14	100
Obsoletus group	Spring	0.6835	70.83	83.33
	Summer	0.6782	68	66.67
	Fall	0.6475	84.21	90
	Winter	0.6407	76.92	57.14
C. pulicaris	Spring	0.6018	55.56	66.67
	Summer	0.5679	77.78	57.14
	Fall	0.6131	40	90
	Winter	0.5465	100	87.5
C. punctatus	Spring	0.7160	82.61	42.86
	Summer	0.6952	66.67	85.71
	Fall	0.6646	65	77.78
	Winter	0.6927	56.25	75
C. newsteadi	Spring	0.6526	78.26	85.71
	Summer	0.6785	50	100
	Fall	0.66	83.33	66.67
	Winter	0.66	76.92	100

The presence/absence probability maps for *C. imicola* obtained from the models are represented in Figure 4.1.

Figure 4.1. – Presence/absence probability maps for *C. imicola* per season.

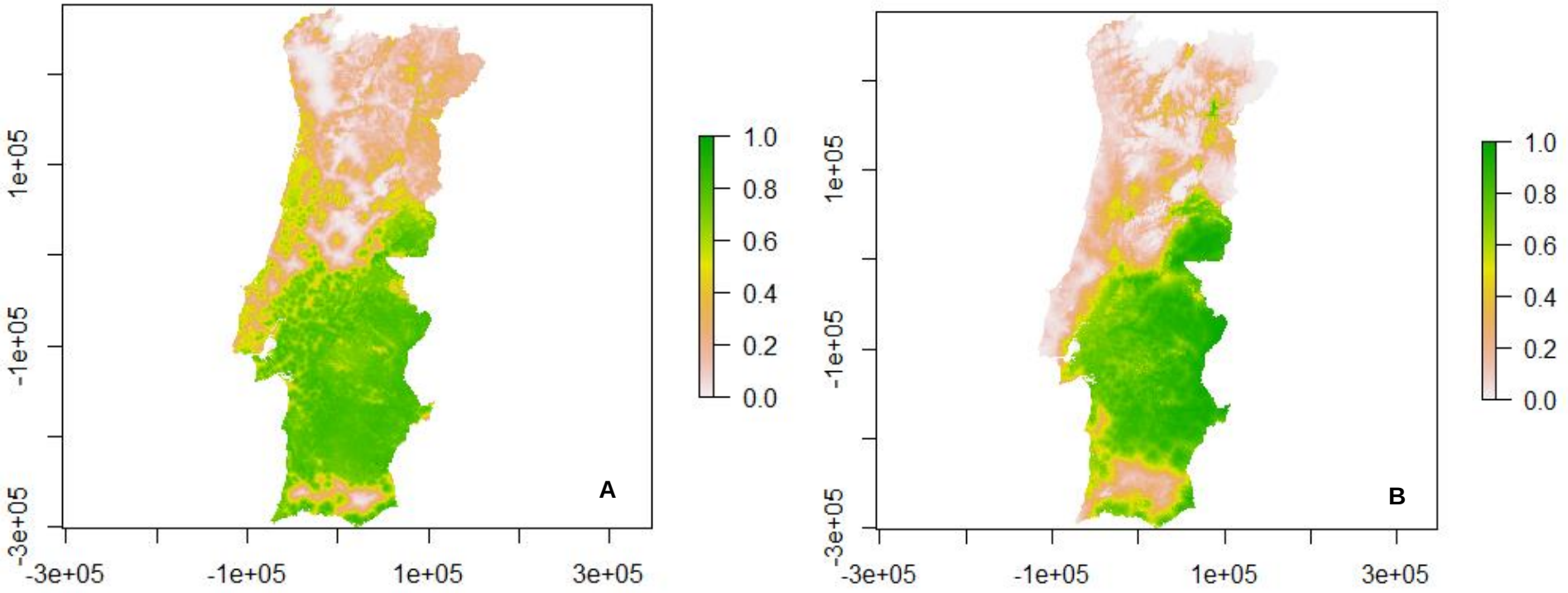
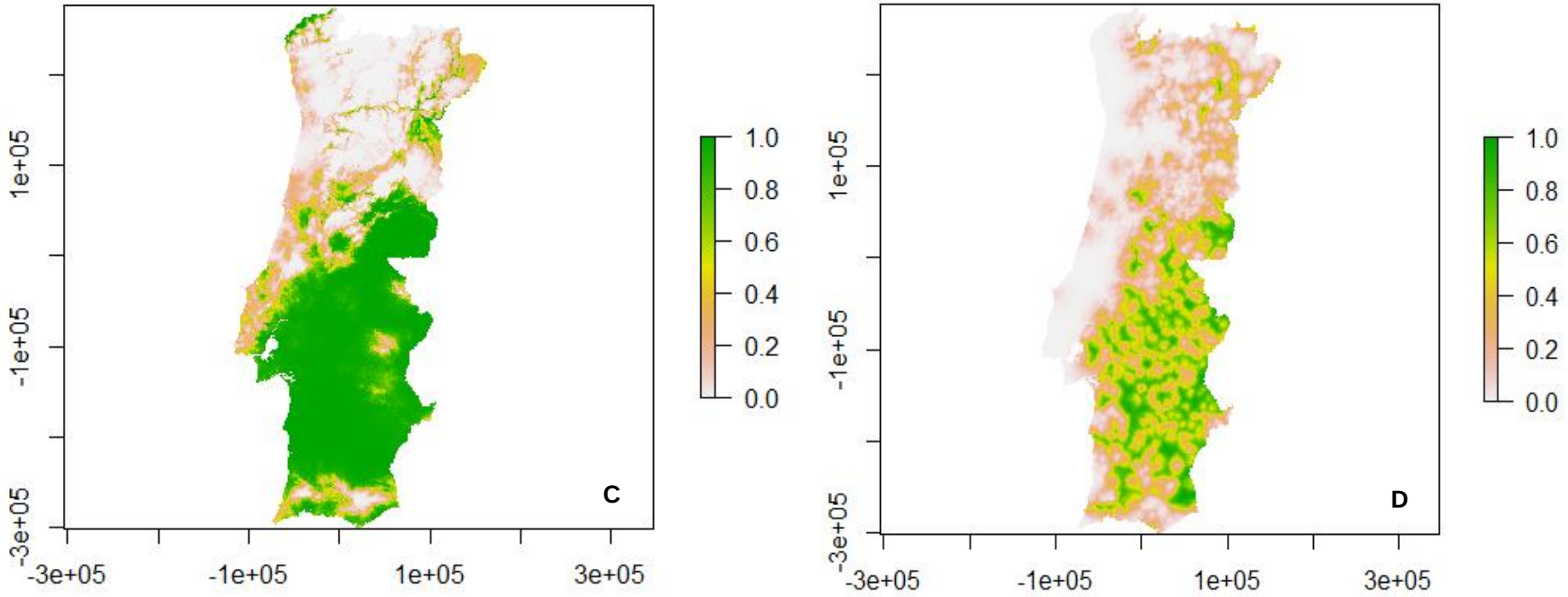


Figure 4.1. – Presence/absence probability maps for *C. imicola* per season (Continuation).



A – Spring; B – Summer; C – Fall; D – Winter.

C. imicola can appear with a high probability in regions below Tagus River and in Beira Baixa from spring to fall, being this probability lower during winter. In the remaining territory, as well as in a small area in South Alentejo and Algarve, the probability for *C. imicola* to be present is very low. *C. imicola* have a higher probability to appear during fall.

The presence/absence probability maps for Obsoletus group species obtained from the models are represented in Figure 4.2.

Figure 4.2. – Presence/absence probability maps for Obsoletus group species per season.

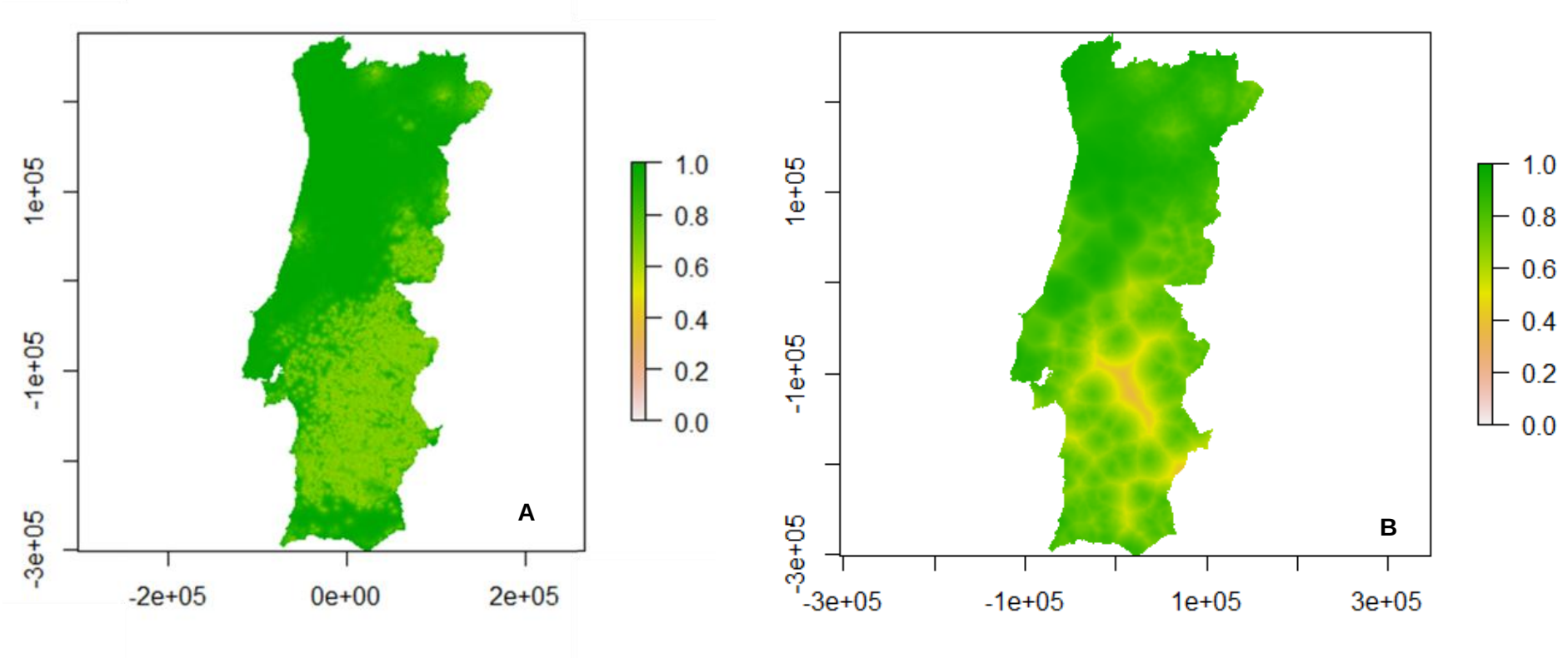
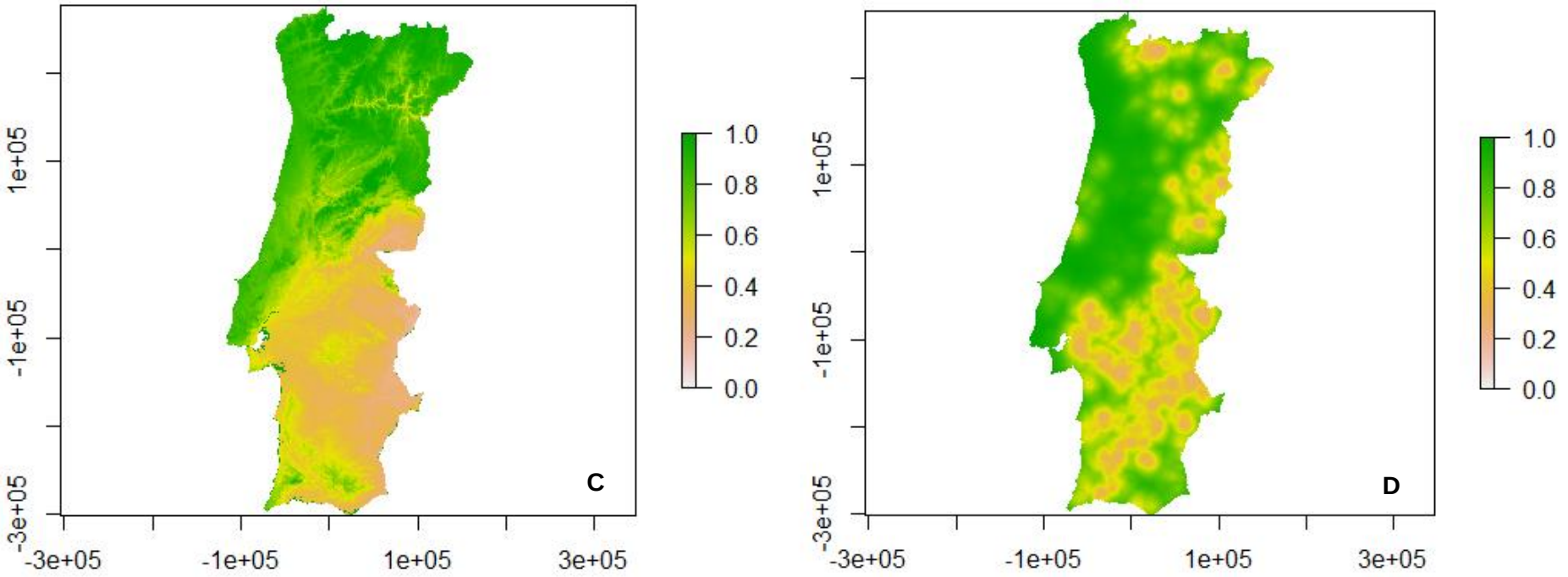


Figure 4.2. – Presence/absence probability maps for Obsoletus group species per season (Continuation).



A – Spring; B – Summer; C – Fall; D – Winter.

Midges from Obsoletus group have a similar distribution in all mainland Portugal in spring and summer, being less probable to find in Centre South region. In fall, the probability to find an Obsoletus group midge falls abruptly below Tagus River, being almost the opposite probability map of *C. imicola* in this season. During winter, Obsoletus group species are less probable to be present in Centre South region and in interior regions of North and Centre North parts of mainland Portugal. *Culicoides* midges from Obsoletus group are more common during spring and summer, in regions above Tagus River (North and Centre North regions).

The presence/absence probability maps for *C. pulicaris* obtained from the models are represented in Figure 4.3.

Figure 4.3. – Presence/absence probability maps for *C. pulicaris* per season.

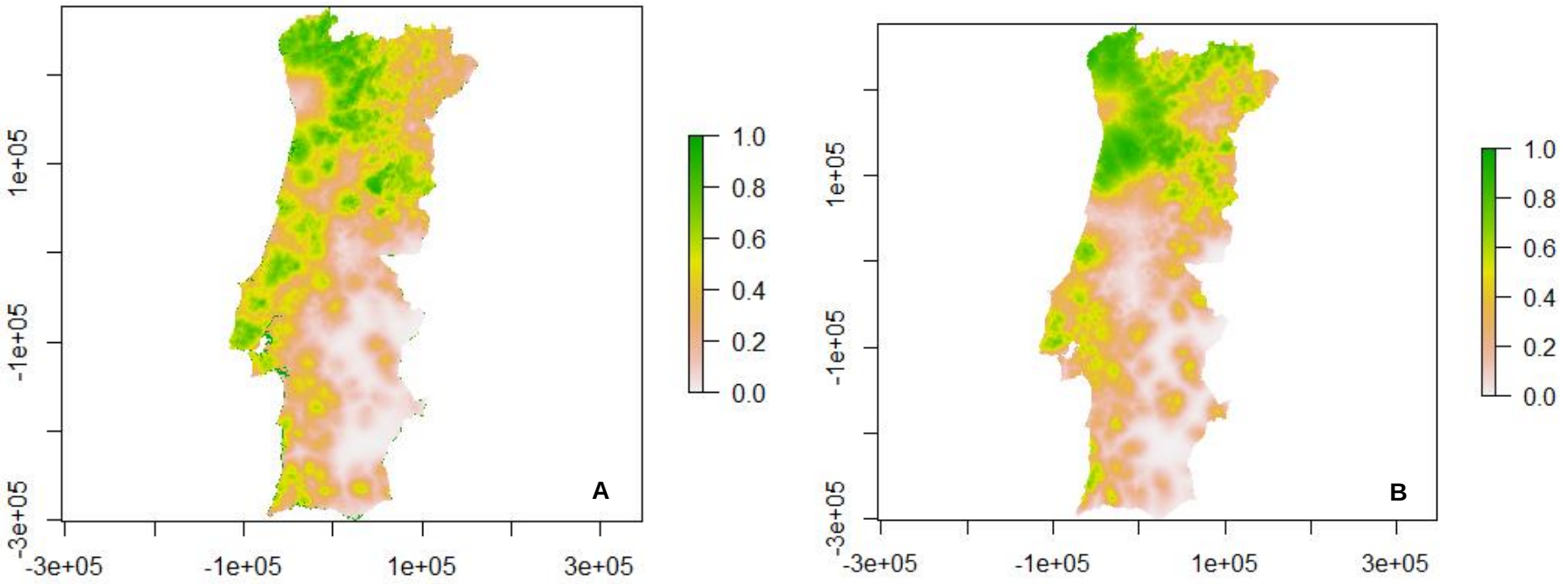
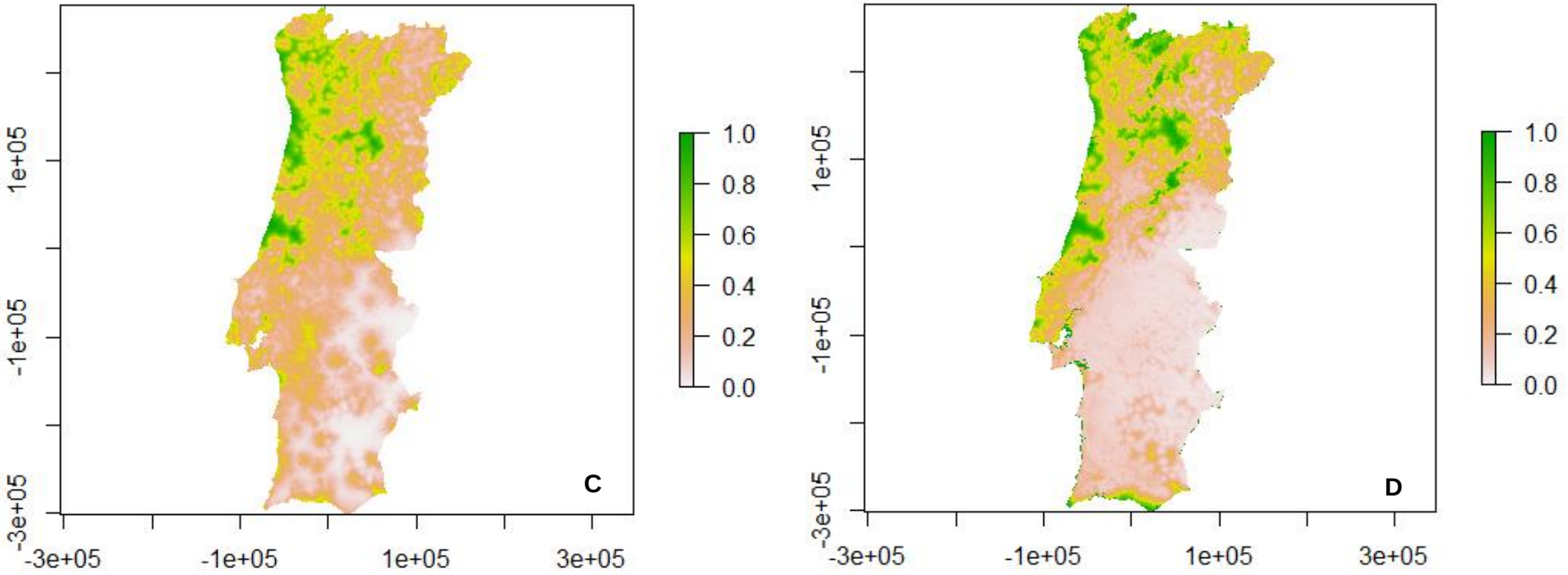


Figure 4.3. – Presence/absence probability maps for *C. pulicaris* per season (Continuation).



A – Spring; B – Summer; C – Fall; D – Winter.

C. pulicaris has a tendency to appear in both coastal and inland regions of mainland Portugal from the North to Tagus River. In Alentejo, the probability to catch this species is extremely low. In spring, it has a high probability to appear from Minho to Aveiro regions. In winter, it concentrates near Atlantic Ocean regions, with the exception of Alentejo coast. In all seasons, *C. pulicaris* tend to appear in high altitude regions from Viseu region and near Serra da Estrela.

The presence/absence probability maps for *C. punctatus* obtained from the models are represented in Figure 4.4.

Figure 4.4. – Presence/absence probability maps for *C. punctatus* per season.

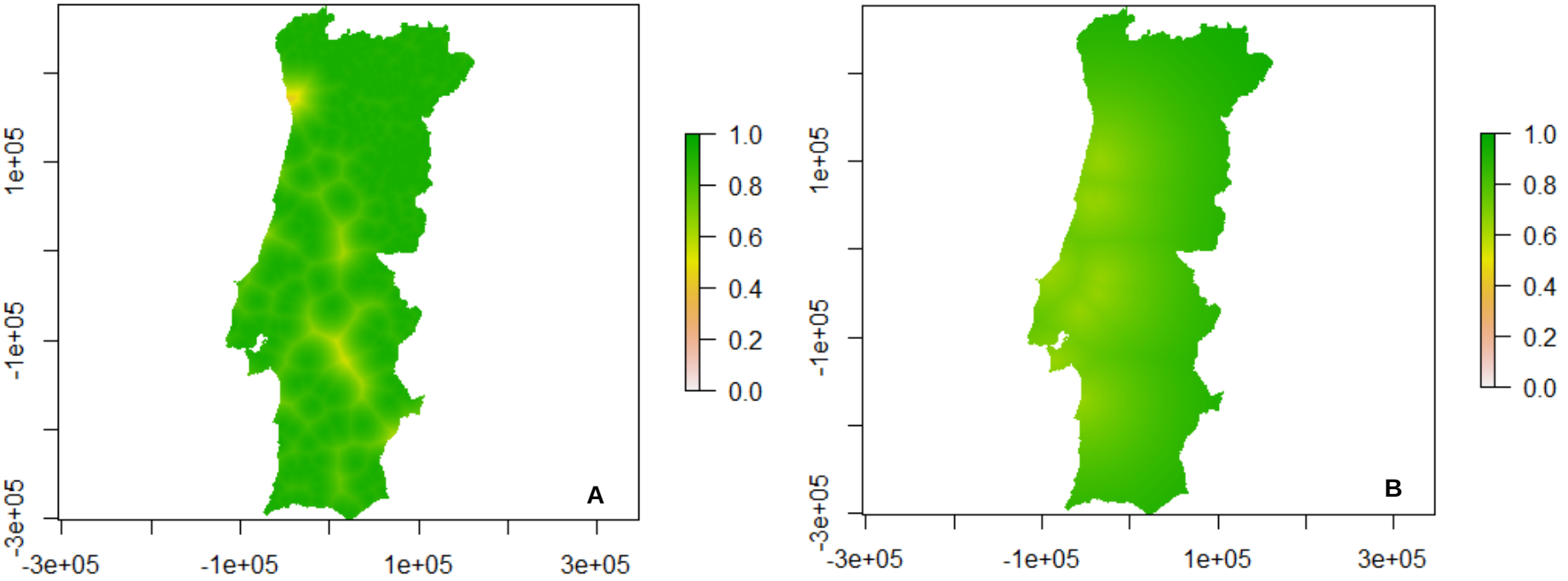
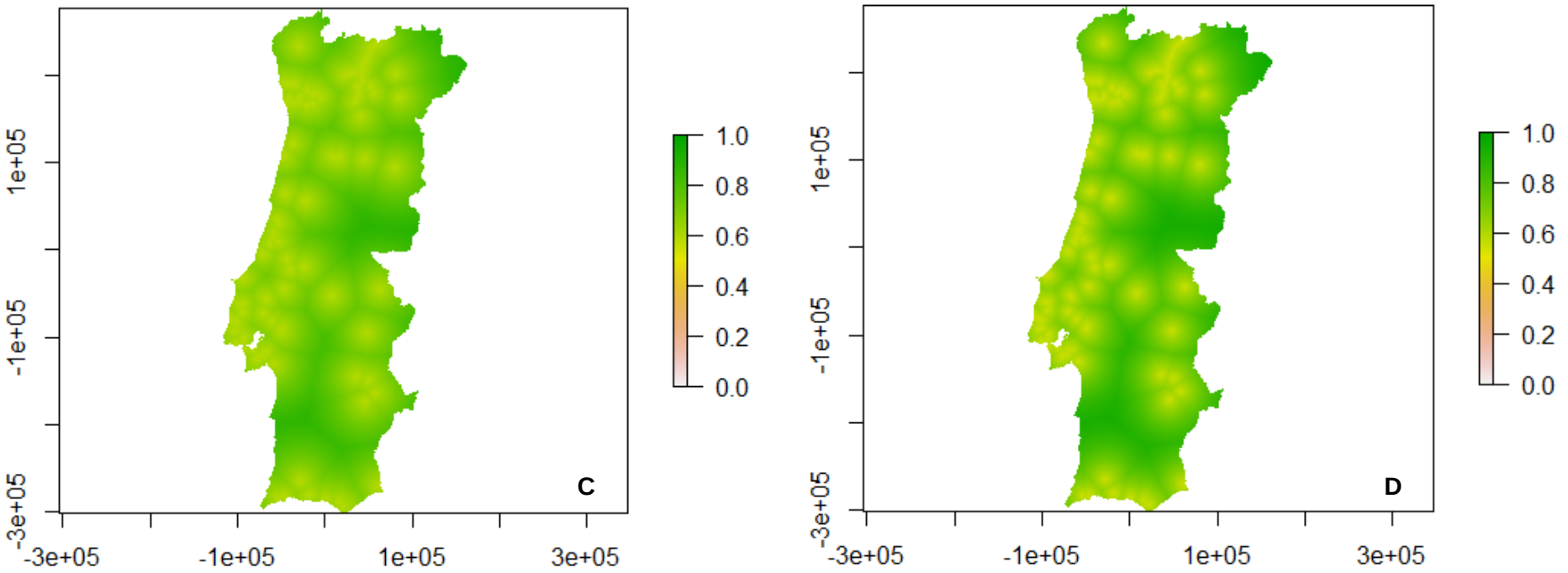


Figure 4.4. – Presence/absence probability maps for *C. punctatus* per season (Continuation).



A – Spring; B – Summer; C – Fall; D – Winter.

C. punctatus is almost equally distributed in mainland Portugal during all year, being less probable to find in Oporto region during spring and being the fall and winter probability maps very similar.

The presence/absence probability maps for *C. newsteadi* obtained from the models are represented in Figure 4.5.

Figure 4.5. – Presence/absence probability maps for *C. newsteadi* per season.

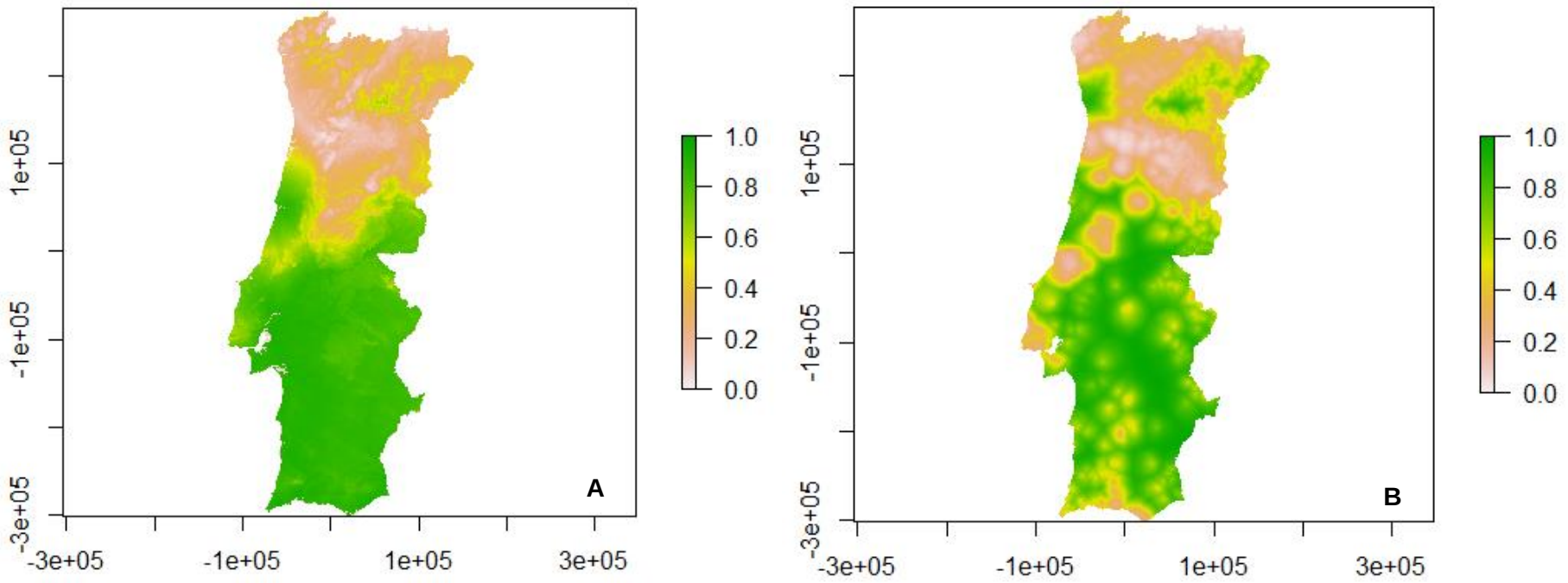
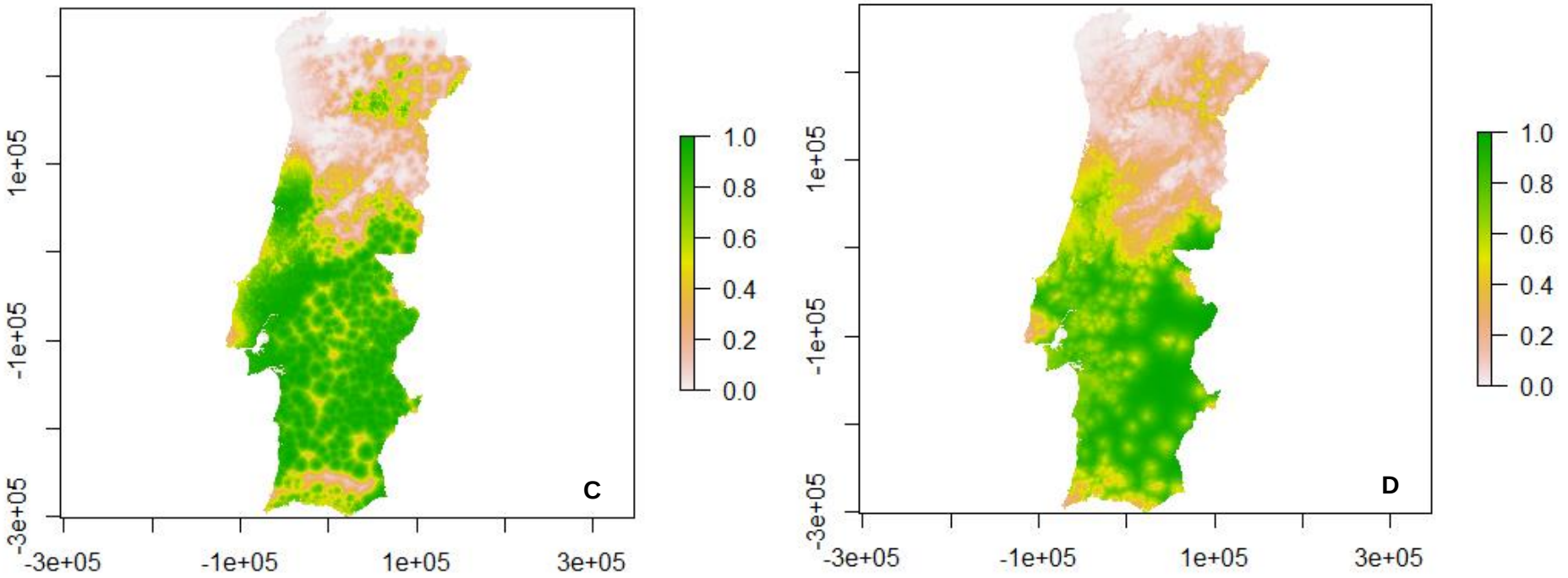


Figure 4.5. – Presence/absence probability maps for *C. newsteadi* per season (Continuation).



A – Spring; B – Summer; C – Fall; D – Winter.

Finally, *C. newsteadi* has a similar distribution in spring, fall and winter, being common in Centre South and South regions, as well as in Beira Baixa and in a coastal area from below Aveiro to Leiria regions, with a high probability in Figueira da Foz. This species is not common in North region during these three seasons. In summer the pattern is different, with a small concentration near coastal areas of Oporto region and near Douro River between Trás-os-Montes e Alto Douro and Beira Alta regions. Although *C. newsteadi* is present during all year, it is more common during spring season.

4.5. Discussion

C. imicola is well adapted to high temperatures and dry environments and its distribution by the entire African continent, Middle East and Southeast Asia is representative of that situation (Wilson & Mellor, 2009). These are the reasons why high mean temperatures are important for this species occurrence during the year in both wet and dry seasons.

The immature stages of *C. imicola* require a semi-moist soil enriched with organic matter but, as the pupae are not able to float on the water surface, such areas cannot be waterlogged (Foxi & Delrio, 2010; de Heredia & Lafuente, 2011; Ippoliti *et al*., 2013). During spring, the existence of non-irrigated arable land favours this species occurrence, probably due to the development of their pupal phases, which do not survive in aquatic environments (Nevill, 1967; Foxi & Delrio, 2010). These conditions restrict the species to flat and slow-draining regions with soils of the clay type (nutrient-rich, water-holding soil) (Conte *et al*., 2007; Ippoliti *et al*., 2013).

As it can be seen from Annex 4.1., this species was not captured with relative humidity below 9% and temperatures above 40.40 °C. The explanation for the permanently irrigated land be favourable for this species development during the summer is the need of a certain percentage of water nearby, since very high temperature (≥40.05 °C) together with elevated dryness (typical from certain zones in Alentejo region, where it appears in this season) is fatal for this species (Conte *et al*., 2007; Guichard *et al*., 2014; Verhoef, Venter & Weldon, 2014). The rapid soil surface layer desiccation has also a negative impact in *C. imicola* occurrence (Conte *et al*., 2007; Ninio *et al*., 2011a), since its absence has been connected to soils with a sandy texture, which are known to have depleted moisture levels (especially in the surface layer) and to lack vital nutrients (Conte *et al*., 2007).

C. imicola breeds in areas where sunny surfaces prevail together with low vegetation (Ippoliti *et al*., 2013). They avoid areas covered by forest and thus differ in this aspect from other European vector species, like those belonging to the Obsoletus complex (Meiswinkel *et al*., 2004b; Conte *et al*., 2007). In mainland Portugal, during fall, non-irrigated arable land, fruit trees, berry plantations, moors and heathlands are suitable for *C. imicola*, showing its preference for different kinds of trees and again for more dry environments, probably for breeding. The negative influence of olive groves in *C. imicola* occurrence shows that this species may have preferences in choosing the best vegetation for breeding, oviposition and for larval and pupae development. Terrains with human involvement (construction sites and discontinuous urban fabric terrains) diminish the risk for this species occurrence.

The appearance of *C. imicola* near water bodies during winter must be further investigated, since these environments could represent a preferential place for this species to overwintering.

C. obsoletus does not seem to breed in waterlogged soils, showing preferences for unsaturated, anthropogenic soil environments in the vicinity of the farms near shaded areas

(Hill, 1947; Kettle & Lawson, 1952; Braverman, Galun & Ziv, 1974; Trukhan, 1975; Mirzaeva, Glushchenko & Zolotarenko, 1976; Mellor & Pitzolis, 1979; Chaker, 1983; Mathieu, 2005; Zimmer *et al*., 2008; Zimmer *et al.,* 2010; Ninio *et al*., 2011b; Zimmer *et al*., 2013a). *C. obsoletus* larvae prefer compost heaps of trees, tree holes (Murray, 1957; Kremer, 1965), broad woodland leaf litter and vegetation, marginal vegetation surrounding open water (Harrup *et al*., 2013), straw contaminated with faeces, organically enriched substrates in stable yards and different types of manure piles (González *et al*., 2013; Harrup *et al*., 2013). The huge diversity of habitat used by *C. obsoletus* can explain its ubiquitous distribution across Europe and development in a great variety of habitats (González *et al*., 2013). *C. scoticus* breeding sites have been largely unknown (Kettle & Lawson, 1952), but they can breed on rotting fungi (Buxton, 1960), ruts of mud (Kremer, 1965) and marshy habitats (Boorman & Goddard, 1970). The immature habitats of *C. scoticus* are not restricted to these ones and they may coexist with *C. obsoletus* (Conte *et al*., 2007). However, *C. scoticus* is unlikely to breed in great numbers in manure, while *C. obsoletus* larvae are present in manure from horses and cows (González *et al*., 2013). Although these two species breed in a great variety of habitats, they show distinct preferences for certain soil conditions: while *C. obsoletus* develops in different types of manure or manure laden soil substrates, *C. scoticus* prefer forest habitats with leaf litter, being more dispersed and less common in light traps (González *et al*., 2013).

Obsoletus group species have preference for shaded areas in pastures near woodlands. This situation can explain why the proximity of agro-forestry areas do not favour the occurrence of these species near the capture points. Probably, species inside this group tend to remain close to their preferable habitats aforementioned instead to search for stabled animals and only feed on them while they are in pastures and not in enclosed stables. Thus, preferential hosts in wild fauna exist and probably substitute farm animals for their blood meals, showing an agreement with previous works (Falconi *et al*., 2011). Talavera *et al*. (2015) also shown that the main vector species for BTV and SBV present on the livestock farms were also present in the neighbouring natural areas with wild ruminants, which would support their putative role as bridge vectors for the transmission of arboviruses between domestic and wild ruminants, in addition to their recognised role as epizootic vectors. The bypass of the pathogen among wild/domestic communities mediated by *Culicoides* bridge vectors (*C. imicola* and Obsoletus group) would facilitate the interseasonal BTV and SBV reintroduction among domestic ruminants.

Agro-forestry areas may be also a potential localization for species inside Obsoletus group to overwinter. This work is also in agreement with other works (Conte *et al*., 2007; Scolamacchia *et al*., 2014), showing that arable lands do not favour this species occurrence. Natural grasslands nearby capture points contribute for these species occurrence, probably due to the presence of animal manure and consequently an optimal environment for larvae

development. As mentioned above, since these species may overwinter in agro-forestry zones, pasture and water bodies do not favour the occurrence of these species near capture points in winter. Concerning meteorological variables, low mean temperatures in both wet or dry fall quarters raises the probability of these species to occur, what is in agreement with these species preferences concerning spatial distribution (mainly in Central and Northern European countries) (Conte *et al*., 2007; Wilson & Mellor, 2009).

C. pulicaris larvae was already collected from molehill soil, silt from the edge of a pond, maize silages reserves, soil in stagnant water, algae and underlying soil, river edges, forest mud, wet grazed field with manure, waterlogged soils near lakes and marshy places and forest leaf litters (Kettle & Lawson, 1952; Konurbayev, 1965; Kremer, 1965; Dzhafarov, 1976; EFSA, 2007; Nevill *et al*., 2007; Kirkeby *et al*., 2009; Zimmer *et al*., 2014).

From the 5 studied species, *C. pulicaris* seems to be less adaptable to our edaphoclimatic environment. Besides the abovementioned places where *C. pulicaris* was collected, it can be pointed out that different kinds of vegetation influence their presence or absence near capture points. Thus, this species tend to remain in their preferable habitats (broad-leaved forest, agro-forestry areas, fruit trees and berry plantations), where they probably feed in wild fauna instead of farm animals or where they overwinter, similarly to Obsoletus group species. This may also justify why *C. pulicaris* is less captured then the other 3 species and those of Obsoletus group. However, is must be referred that mixed forest, natural grassland and pastures nearby the capture points also raises the probability for this species occurrence, probably due to their different and specific ecological characteristics, which must be further investigated. Additionally, this species has a preference for public, industrial or mine dump sites, which may justify partially the occurrence of this species near urban areas, probably for larval and pupal development and also to perform blood meals in humans, as reported by other authors (Santiago-Alarcon *et al.,* 2012). This species occurrence is favoured by lower mean temperatures during the driest quarter of spring and winter seasons, just like Obsoletus group species. This fact justifies the similar spatial distributions across Europe when compared with Obsoletus group species (Fauna Europaea, 2015).

C. punctatus larvae have been found in the same places as *C. pulicaris*, in open marshy fields (Boorman, 1989), in sludge samples, with or without organic matter, and animal manure together with *C. imicola* (Pena, 2003), in wet soil between silage reserves (together with *C. stigma*), soil in stagnant water, algae and underlying soil (Zimmer *et al*., 2014) and silt from a pond (Zimmer *et al*., 2013b; González *et al*, 2013).

C. punctatus, contrary to *C. pulicaris*, is the most well adapted species to our edaphoclimatic environment. This species is present all year long and only few variables favour or not its occurrence near capture sites. These characteristics show that this species gathers all the ecological conditions to be a potential vector species of BTD. Most importantly, it can be observed that *C. punctatus* occurrence is not favoured by human presence, due to

construction sites. However, this study has shown that inland marshes do not favour this species occurrence (at least during summer), what is in contradiction with previous works (Boorman, 1989; Zimmer *et al*., 2014) and, so, further studies must be performed to gather some conclusions concerning this point.

Like for most of the *Culicoides* species, the breeding sites of *C. newsteadi* are also poor known. This species was found breeding in shallow, brackish pools, lined with decaying vegetable material (Kettle & Lawson, 1952; Foxi & Delrio, 2010). Pena (2003) recovered this species in mud samples.

Finally, *C. newsteadi* is influenced by higher mean temperatures during the wettest quarter of several seasons, like *C. imicola* species. *C. newsteadi* shows an intermediate distribution between *C. punctatus* and *C. pulicaris* and since it also occurs all year, it gathers all the ecological conditions to be a potential vector species of BTD, as pointed out by other authors (Foxi & Delrio, 2010; Goffredo *et al*., 2015). Several types of vegetation favour this species appearance, like agro-forestry areas, olive groves, together with water courses. However, natural grasslands and coniferous forests do not favour its occurrence. Thus, this species appear to have different vegetation preferences, just like happens with *C. imicola*. At last, discontinuous urban fabric terrain favour this species occurrence during fall, showing that *C. newsteadi* also have an anthropophagic (and probably endophagic) behaviour during blood meal, as shown by other works (Braverman, Boreham, Galum & Ziv, 1977; Slama, Haouas, Mezhoud, Babba & Chaker, 2015).

All of this five species can make multiple generations (multivoltine) along the year, since they are captured in all seasons. This work shows that these species can live long enough to acquire, maintain and transmit different diseases (like BTD, SBD and AHSD) to their hosts. Thus, further biological studies with *C. punctatus* and *C. newsteadi* species must be performed to efficiently reveal the role of these species in BTD transmission (Foxi & Delrio, 2010; Goffredo *et al*., 2015).

Modification of breeding sites can be made at the local scale or in a bigger area. Sometimes, this modification can reduce breeding sites and be an impediment for larvae development. On the local scale, a reduction of potential breeding sites can be executed through their spatial identification and removal by an adjustment of farm management, including: the avoid of overflowing cattle troughs, dripping taps or reducing manure amount and dung piles directly on the farm (Lüken *et al*., 2014). To be implemented in a bigger area, this breeding site modification includes complex drainage systems and habitat elimination, which require specialized machinery (de Heredia & Lafuente, 2011). Similarly, proper maintenance of ponds, lagoons, water accumulations and scheduling of irrigation systems can influence the reduction of adult populations of some species (Mullen & Durden, 2009). The prevention of water holes overflow, rainwater accumulations and drainage of channel holes are simple actions that can have a positive impact on the control of species belonging to Obsoletus

group (de Heredia & Lafuente, 2011), although these two last actions can cause severe ecological problems (*e.g.*, by reducing species diversity) (Lu *et al*., 2009). Coverage of big heaps of manure deposited occasionally using tarpaulins prevents the access of mosquitoes and therefore they cannot breed. Furthermore, drying manure with compost bins is also a good option to avoid *C. obsoletus* breeding (Carpenter *et al*., 2008c). Elimination of silage residues, leftover feed along the feed bunk, and dung adhering to walls (Zimmer *et al*., 2010, 2013a, 2013b) are hygienic measures that can be undertaken in farms in order to reduce biting midges populations. However, a comprehensive understanding on the breeding ecology of *Culicoides* biting midges is still missing (Lüken *et al*., 2014).

Data collected for the remaining species shows the limits concerning meteorological conditions (minimum and maximum temperatures, wind speed and relative humidity) registered when a specific *Culicoides* species was caught, as well as the means for each variable.

It must be pointed out that some species were caught at very low temperatures (9 different species below 0 °C): *C. imicola*, *C. obsoletus*, *C. montanus*, *C. pulicaris*, *C. punctatus*, *C. newsteadi*, *C. gejgelensis*, *C. univittatus* and *C. subfasciipennis*. Since these species are resistant to sub-zero temperatures, they must be further studied concerning overwintering of different arboviruses and the potential role in the transmission of VBD to susceptible hosts of those considered as “non-vector” species.

On the other hand, several species were caught at very high temperatures (16 different species above 40 °C). No species were caught when relative humidity was below 9% and 47 different species were caught with relative humidity above 90%. This information shows that most of the *Culicoides* species captured can survive between a wide range of temperatures and relative humidity. Complementary information given in Annex 4.1. can be useful to further studies concerning *Culicoides* ecological preferences.

After elaboration of the different dispersion maps, concerning the probability to capture the referred species, it is clear that all of them have different distributions in mainland Portugal.

C. imicola species are concentrated in regions below Tagus river and in Beira Baixa, an inland area in Central North region. The low probability observed in a small region between Baixo Alentejo and Algarve is probably due to the very dry climate and hot temperatures registered in this region, especially in summer. It is very important to refer that, during fall, *C. imicola* has the capacity to disperse further North than what is observed during other seasons. It should mentioned that, although the estimated cut-off point for this season was high (0.6864, with a mean sensibility of 95% and a mean specificity of 100%), the risk for this species to appear in Minho and Trás-os-Montes e Alto Douro regions is considerable and real (Figure 4.1.), as it was pointed out in previous works for this last region (Capela *et al*., 2003; Pena, 2003).

The geographical range of *C. imicola* appears primarily limited by cold stress and dry stress, and to a lesser extent wet stress (Guichard *et al*., 2014). In Spain, *C. imicola* has established itself in all southwest (near the Portuguese frontier) and central regions of Madrid province. In the Mediterranean zone it has been found on the coast of Catalonia, as well as in areas of Alicante and Murcia (de Heredia & Lafuente, 2011). It is also a very abundant species in the Balearic Islands (Miranda, Borràs, Rincón & Alemany, 2003). In other communities, especially in Castile and North zone, there have been sporadic incursions of specimens which do not prevail (de Heredia & Lafuente, 2011), just like those observed by other authors in Portuguese territory (Capela *et al*., 2003; Pena, 2003; Ribeiro *et al*., 2015). The large scale distribution pattern seems to be strongly influenced by the requirements of the species for high summer temperatures and dry summer conditions (Calvete *et al*., 2008). However, it is possible that *C. imicola* is expanding its range northwards, maybe due to climate changes (de Heredia & Lafuente, 2011), although several factors, such as dispersal abilities, size of the source population, meteorological conditions and the presence of natural barriers, limit the colonization of those regions (Guichard *et al*., 2014).

Midges from Obsoletus group demonstrate a high tolerance for a wide range of temperatures, altitudes and terrain slopes, having a broad distribution in European continent (Conte *et al*., 2007). Species of the Obsoletus complex occur only within the temperate and boreal ecozones (Schultz, 2005) of the Holarctic region (includes Palearctic and Nearctic ecozones), although some species penetrate southwards into the northern half of the Mediterranean region (Meiswinkel *et al*., 2014b).

Species from Obsoletus group prefer Northern mainland Portugal regions, being almost absent in Centre South regions during fall and winter, which is compatible with results obtained in Chapter 3. Although *C. imicola* and Obsoletus group species have some overlapping or common areas to appear, they have different preferences in fall and winter seasons, being their occurrence in mainland Portugal almost the opposite. This distribution has been explained by the fact that species belonging to Obsoletus group requires areas with relatively low annual average temperature and high soil moisture (Purse *et al*., 2004b). These results are in line with Calvete *et al*. (2008), who described a similar latitudinal abundance pattern for livestock farms on the Iberian Peninsula. While *C. imicola* is present in the warmest zones, species from Obsoletus group are present in those with relatively low mean annual temperatures (de Heredia & Lafuente, 2011).

C. pulicaris is well distributed by several European countries, being its geographic range similar to species from Obsoletus group (de Heredia & Lafuente, 2011; Fauna Europaea, 2015). This species has a preference for Northern areas of mainland Portugal, being more probable to find in coastal regions and near high altitude zones. However, Pena (2003) referred the presence of this species mainly in the Northeast and in the Southeast regions of mainland Portugal, near the Spanish frontier. Since this species is also dispersed by all

Spanish territory, although with low abundance, and being more common in the South zone of Iberia than the North (de Heredia & Lafuente, 2011), probably it is not fixed to a specific ecosystem, being present in several types of edaphoclimatic conditions.

Although *C. newsteadi* can be found in several European countries, it is absent in several central and east countries of the same continent (de Heredia & Lafuente, 2011). Pena (2003) captured *C. newsteadi* in traps located near Douro River, Coimbra, and in Centre South and South regions. Our results showed that *C. newsteadi* follows *C. imicola* species pattern, although it can be found in more Northern areas than this last one, being in agreement with Pena (2003).

Finally, *C. punctatus* can be found in Palearctic ecozone until Mongolia, Near East and North of Africa, as well as in the African Tropical zone. *C. punctatus* is well distributed by all countries in Europe, from Ireland to Russia (de Heredia & Lafuente, 2011). Our results showed that this species is dispersed by all country, being in agreement with Pena (2003). *C. punctatus* has a probability of 50% or more to occur in any part of mainland Portugal in almost all seasons, being very well adapted to our climate.

Scolamacchia *et al.* (2014) have shown that *C. pulicaris*, along with its very low abundances in certain areas of Netherlands, is also unable to penetrate into all areas and to satisfy the three inter-related elements (abundance, seasonal persistence and multivoltinism) that a competent vector appears to possess, unlike biting midges of *C. punctatus* and *C. newsteadi*, which appear all year and gather all the conditions mentioned above. The recent findings of BTV genome in parous females of these two last species (Goffredo *et al*., 2015) must be taken into account, since they can be potential vectors of this disease.

Knowledge of suitable breeding sites of each species, particularly those implicated in transmission of parasites or pathogens, is essential to evaluate the risk in an area and therefore to contribute to the development of integrated control strategies. Proper manure management, perhaps coupled with extensive trapping using odour attractants sufficiently potent and/or insecticide-treated targets for *Culicoides* midges could reduce midge populations in localized or isolated areas and therefore reduce the possibility of arbovirus transmission (González *et al*., 2013).

Although these results should be taken with caution, since the lack of basic knowledge concerning *Culicoides* ecology can lead to uncertainties in modelling results, as pointed out by Lühken *et al*. (2014), they should be observed as an extremely important auxiliary information to control measures that should be taken in order to reduce the risk of VBD outbreaks and to understand where other species can also act as vectors of such diseases.

Chapter 5: Morphological modifications in *Culicoides* sensorial organs exposed to pyrethroid insecticides

5.1. Introduction

Visual stimuli and body odour are the major elements involved in host attraction of adult *Culicoides* (Bishop, McKenzie & Spohr, 2008). Host visibility and characteristics (*e.g.*, shape, colour, size and contrast) may affect the attractiveness of some *Culicoides* species to the hosts (Humphreys & Turner, 1973; Tanner & Turner, 1974; Koch & Axtell, 1979; Braverman, Rechtman, Frish & Braverman, 2003; Bishop *et al*., 2008).

Culicoides have olfactory structures in antenna and palpus for the detection of volatile compounds and thus host location (Bowen, 1991; Gibson & Torr, 1999; Grant & Kline, 2003; Logan & Birkett, 2007). A diversity of host-derived products are perceived by olfaction (*e.g.*, CO_2, octenol [1-octen-3-ol], lactic acid, phenols, acetone) and may attract hematophagous insects (Nelson, 1965; Kline, Hagan & Wood, 1994; Ritchie, van Essen, Kemme, Kay & Allaway, 1994; Blackwell, Dyer, Mordue, Wadhams & Mordue, 1996; Gibson & Torr, 1999; Bhasin, Mordue Luntz & Mordue, 2000; Marquardt, Demaree & Grieve, 2000; Braverman, Wegis & Mullens, 2000; Cilek & Kline, 2002; Grant & Kline, 2003; Mordue, 2003; Mands *et al*., 2004; Logan & Birkett, 2007; Harrup *et al*., 2012). *Culicoides* show more attraction to greater sized hosts probably due to a higher quantity of CO_2 released by the host (Humphreys & Turner, 1973; Tanner & Turner, 1974; Raich *et al*., 1997). The host search process by olfaction is influenced by several factors like solar radiation, wind speed, temperature and humidity, since they limit *Culicoides* fly activity (Bhasin, 1996; Blackwell, Wadhams & Mordue, 1997).

In attempts to control *Culicoides*-borne arboviruses, such as BTV and AHSV outside of their endemic range, enforced vaccination campaigns and livestock movement restrictions are usually employed as the most effective way of controlling outbreaks (Savini *et al*., 2008). Where safe and effective vaccines to *Culicoides*-borne viruses are either not initially available or economically unviable, control measures against *Culicoides* have been recommended by veterinary authorities to reduce host-vector contact and thus alleviate arboviruses transmission (Venail *et al*., 2015).

The use of insecticide residual spraying within stables and during transport when livestock is moved outside a restricted movement zone has been recommended in protecting animals with high economic value (Schmahl *et al*., 2009c; Venail *et al*., 2015). Additional physical measures have also been suggested to reduce *Culicoides* populations such as the mechanical removal and/or reduction of larval breeding sites on farms and housing livestock during periods of high *Culicoides* activity (EFSA, 2008).

To date, no insecticidal products have been authorized specifically against *Culicoides* biting midges in the European Union, although a wide range of products are available, licensed and in use against other arthropods of veterinary importance (EFSA, 2008). Worldwide, the most commonly used method to protect livestock from *Culicoides* is the application of insecticides to livestock at risk of infection. Synthetic pyrethroid active ingredients (alpha-cypermethrin,

deltamethrin and permethrin) are most often used in this role, but certain organophosphate products (diazinon/dimpylate and phoxim) are also still available and licensed for use in European continent (Carpenter *et al*., 2008c; Venail *et al*., 2015). Permethrin and deltamethrin are two of the more frequently used insecticides in vector and pest control (Venail, 2015).

Pyrethroids insecticides are widely used to control a broad range of insect-pests in agriculture, public health, veterinary medicine and residential settings, accounting for about 25% of the worldwide insecticide market (Casida & Quistad, 1998). However, there has been little information about the strategies taken for controlling *Culicoides* bites and the impact of these interventions on prevention and control of the vector and, consequently, BTV, since the appearance of the disease in European territory in 1998 (Carpenter *et al*., 2008c).

Although different kinds of molecules have been used to prevent *Culicoides* bites (Carpenter *et al*., 2008c; Schmahl *et al*., 2009c; Venail *et al*., 2015), new studies with the same molecules at lower concentrations are of major interest, since insecticides have a huge negative impact in the environment, user health and in animal products, together with emerging resistances by different types of parasites to these kinds of molecules (Carpenter *et al*., 2008c; Rinkevich, Du & Dong, 2013; Zhu *et al*., 2013). This risk exists considering that products based on single classes of insecticide have been used on a wide scale on livestock to control other arthropods including ticks, horn flies and stable flies in addition to often being used on crops (Venail *et al*., 2015). Thus, the main goals of these studies are to obtain the optimal concentrations to prevent midges bite and the spread VBD, improving animal welfare.

5.2. Objectives

The main objectives of this study were:

1) To investigate, using SEM, the morphological modifications of sensorial structures (sensilla basiconica of the 3rd palpus segment) of dead and alive *C. imicola* midges after their exposure to permethrin at different concentrations (0.01%, 0.05% and 0.1%), comparing to a control group.
2) To investigate, using SEM, the morphological modifications of sensorial structures (sensilla basiconica of the 3rd palpus segment) of dead and alive *C. imicola* midges after their exposure to deltamethrin at different concentrations (0.0001%, 0.00025% and 0.001%), comparing to a control group.

5.3. Materials and methods

The procedures mentioned in the 5.3.1., 5.3.2. and 5.3.3. sections were exclusively performed by Roger Venail (Entente Interdépartementale pour la Démoustication [EID] du littoral méditerranéen, Montpellier, France) and collaborators (see Venail *et al*., 2015).

The procedures referred in 5.3.4. section were performed in CIISA, FMV-ULisboa and FCUL with the same midges used in the abovementioned sections, which were kindly provided by Roger Venail.

5.3.1. *Culicoides* collection and identification

Briefly, field populations of *C. imicola* were collected from two regions: Piannotoli Caldarello, Corsica Island, France (date of collection: August, 2012) and Caldes de Malavella, Catalonia, Spain (date of collection: September, 2012). Collection sites were privately owned farms characterized by abundant populations of *Culicoides* target species and reduced use of insecticides on the animals or pesticides on crops.

C. imicola biting midges were collected using a modified suction UV light trap (OVI model, South Africa) (Venter *et al*., 2009) with the collection beaker replaced by a fine mesh netted cage to enable live collections. To prevent desiccation of *Culicoides* during the collection period, wet paper was placed on aluminium foil and rolled around the mesh cages. Traps were set before sunset and retrieved at dawn.

Culicoides collection cages were stored in an isothermal container with an ice pack during transport to the insecticide trials room. Following completion of insecticide trials, *C. imicola* were identified (Delécolle & De La Rocque, 2002) using a binocular microscope (Venail *et al*., 2015).

5.3.2. Selection of insecticides and production of impregnated papers

Insecticide active ingredients were selected from those used most frequently in pour-on formulations within Europe: alpha-cypermethrin, deltamethrin, permethrin, chlorpyriphos-methyl, phoxim and diazinon (Venail *et al.*, 2015). All active ingredients were used at > 98% purity (Pestanal®, a registered trademark Sigma-Aldrich Laborchemikalien Gmbh, London, UK).

Test papers (Whatman n.º 1 filter paper, 90 g/m^2, 12 x 15 cm) were impregnated following training provided by a WHO collaborative centre (Laboratoire de Lutte Contre les Insects Nuisibles, Institut de Recherche pour le Développement, France). Insecticide active ingredients were applied at different concentrations to papers in a silicone oil as the carrier agent (2 ml per paper, 67% acetone and 33% silicone oil). Control papers were impregnated with 2 ml of acetone-silicone oil mix only. Impregnations were conducted by Roger Venail to ensure consistency.

Papers were impregnated a few days before the testing period, wrapped in aluminium foil and then stored at 4 °C. Impregnated papers were sent to each country in a polystyrene cooler box with ice cooler packs for maintaining the temperature at 4 °C during transport. Each paper was used three times in assays and stored at 4 °C between trials (Venail *et al*., 2015).

5.3.3. Insecticide susceptibility tests

Insecticide susceptibility tests were performed following the standardized WHO protocol for adult mosquito bioassay using test tubes (WHO/VBC/81.806) (WHO, 1981) adapted for *Culicoides* (Venail *et al*., 2011).

For field collected *C. imicola*, only unpigmented females that were believed to have not previously taken a blood meal were used in data analysis, as determined through observation of abdominal pigmentation (Dyce, 1969), since insecticide susceptibility could be age specific (Rajatileka, Burhani & Ranson, 2011) and both physiological status and sex dependent (Chareonviriyaphap *et al*., 2006; WHO, 2013).

Briefly, *C. imicola* biting midges were exposed for 1 h to either insecticide-impregnated papers or a control paper with the carrier compound only. For each replicate carried out with *C. imicola*, approximately 100 unsorted individuals were placed in each tube. Following this exposure period, all *Culicoides* (including incapacitated individuals) were transferred from exposure to observation tubes. Observation tubes were then stored vertically for 24 h and *Culicoides* within tubes were given access to a 10% sugar solution. Following the observation period, live and dead individuals were recorded and placed in 96% ethanol.

A replicate within the trials consisted of one complete set of serial dilutions and one negative control (untreated paper). When the percentage of dead *C. imicola* on control was higher than 20%, all the replicate was excluded, and when it was between 5 and 20%, an Abbott correction was performed (Venail *et al*., 2015).

5.3.4. Specimens preparation for SEM

At the time of this study, only permethrin and deltamethrin insecticide susceptibility tests at lower concentrations (permethrin: 0.01%, 0.05% and 0.1%; deltamethrin: 0.0001%, 0.00025% and 0.001%) had been performed by Venail and collaborators.

A total of 150 *C. imicola* submitted to the insecticide susceptibility tests (permethrin and deltamethrin at the referred concentrations) were kindly provided by Roger Venail.

The live and dead individuals that were placed in 96% ethanol (n=101) (section 5.3.3.) were washed with 70% ethanol and fixed in 2.2% glutaraldehyde (Panreac, ref. 163857.1611) in sodium cacodylate buffer (Sigma Aldrich, ref. 70114). Afterwards, dehydration was performed with acetone (series from 70% to 100%). Specimens were dried using critical point drying method in a JEOL-JFC-1200, mounted in stubs and coated with gold-palladium (Sigma Aldrich, ref. 716928) (Alexandre-Pires *et al*., 2010). Subsequently, *C. imicola* were observed in a JEOL-JSM-5200LV electronic microscope. The accelerating voltage ranged from 10-25 kV.

On a first approach, due to an easier visualization when compared with antennal and abdominal sensilla, sensilla basiconica of the 3rd palpus segment were morphologically evaluated and compared with control individuals not exposed to the active ingredients.

The concentrations of the different active ingredients, as well as the number of *C. imicola* analysed with SEM, are referred in Table 5.1.

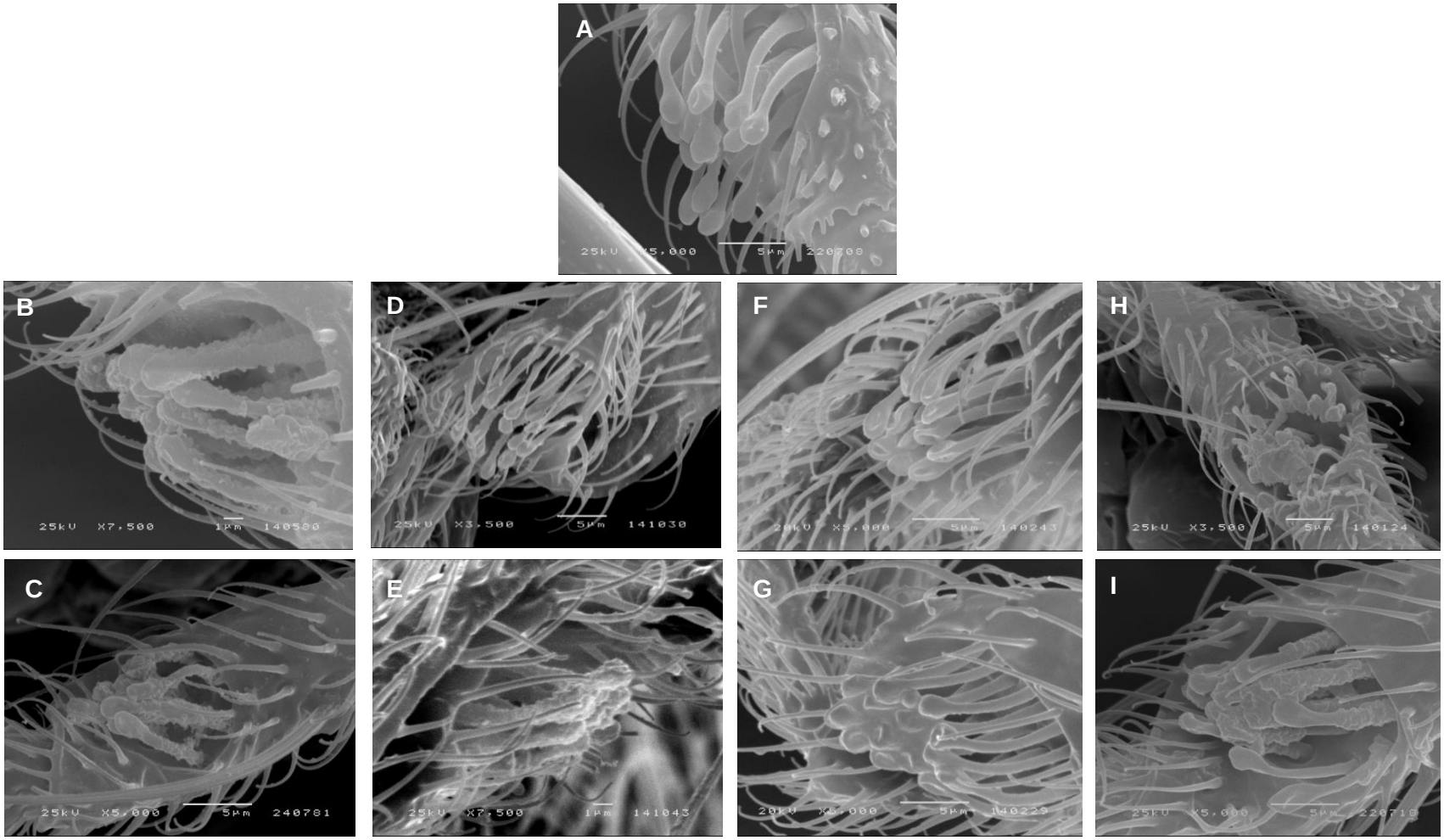
Table 5.1. – Concentrations of deltamethrin and permethrin at which *Culicoides imicola* specimens were submitted and number of analysed specimens with SEM (n=101).

Active ingredient	**Concentrations (%)**	**Number of midges analysed (SEM)**
Deltamethrin	Control	26
	0.0001	7
	0.00025	5
	0.001	17
Permethrin	Control	28
	0.01	6
	0.05	4
	0.1	8

5.4. Results

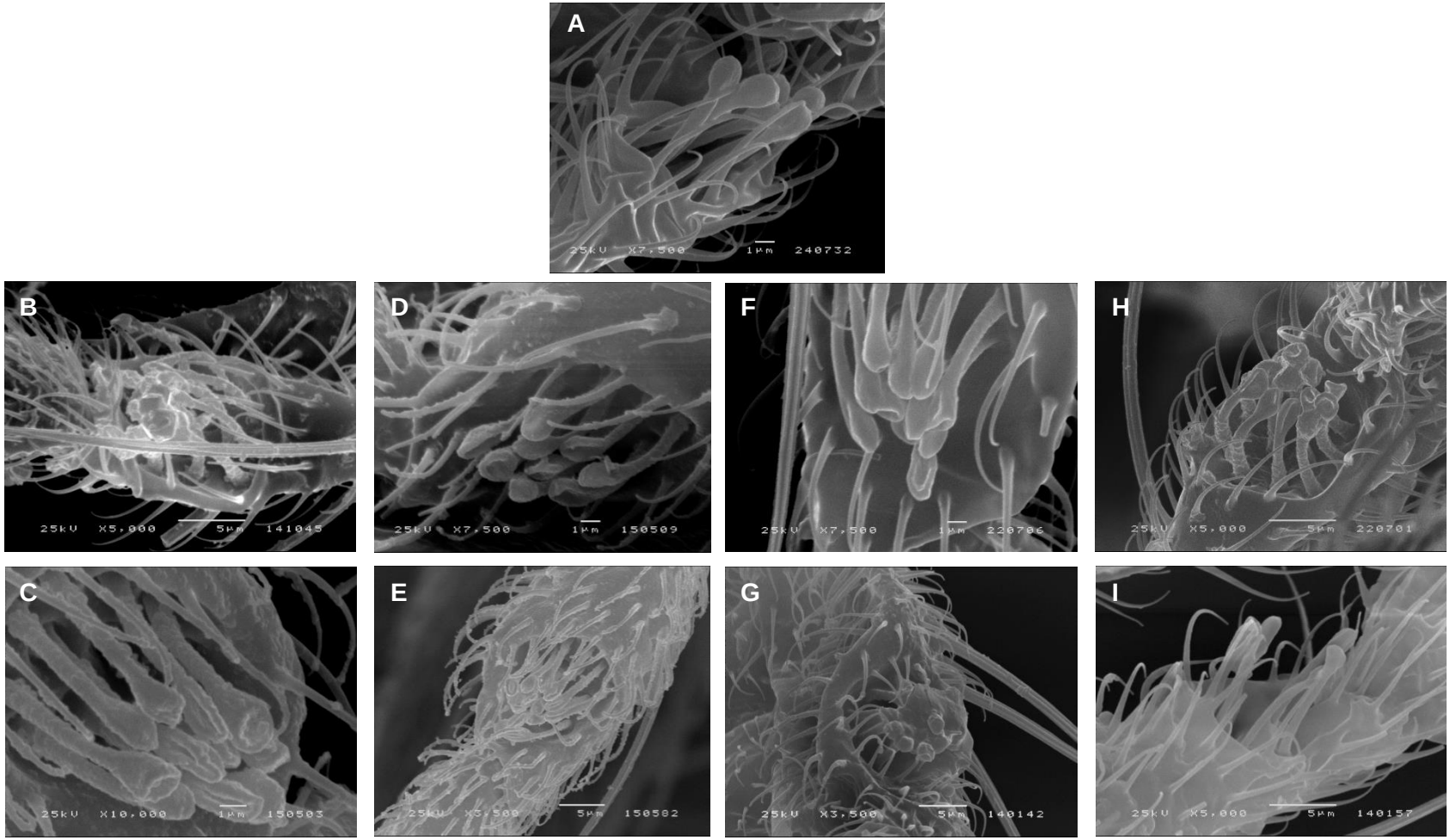
Morphological modifications in sensilla basiconica of the 3rd palpus segment of 101 *C. imicola* specimens randomly selected were morphologically evaluated and those submitted to insecticides (n=47) were compared with control individuals (n=54) not exposed to the active ingredients at different concentrations (Figures 5.1. and 5.2.).

Figure 5.1. – *Culicoides imicola* from control group and those submitted to different concentrations of deltamethrin active ingredients (after 24h of exposure). Photos by courtesy of Alexandre-Pires, 2013-2015. (Photos A, F, G and H: Ramilo *et al*., 2014)



A – *C. imicola* from control group; Midges submitted to deltamethrin 0.0001% that survived (B) and died (C) after exposition; Midges submitted to deltamethrin 0.00025% that died (D and E) after exposition; Midges submitted to deltamethrin 0.001% that survived (F and G) and died (H and I) after exposition.

Figure 5.2. – *Culicoides imicola* from control group and those submitted to different concentrations of permethrin active ingredients (after 24h of exposure) Photos by courtesy of Alexandre-Pires, 2013-2015. (Photos G and I: Ramilo *et al*., 2014).



A – *C. imicola* from control group; Midges submitted to permethrin 0.01% that survived (B) and died (C and D) after exposition; Midges submitted to permethrin 0.05% that survived (E) after exposition; Midges submitted to permethrin 0.1% that survived (F, G and H) and died (I) after exposition.

Midges from control group exhibited a normal conformation of sensilla basiconica: individualized disposition of structures, without rigidity and a rounded shape on their anterior extremity.

All concentrations of both molecules induced morphological modifications in, at least, one of the sensilla basiconica of the 3rd palpus segment (Table 5.2.), with different types of conformation.

Table 5.2. – Number of *C. imicola* specimens with morphological modifications in the sensilla basiconica of one or both 3rd palpus segment concerning different concentrations of deltamethrin and permethrin.

Active ingredient	Concentrations (%)	Both palpus		One palpus	
		Alive	Dead	Alive	Dead
Deltamethrin	0.0001	5	2	0	0
	0.00025	1	3	0	1
	0.001	8	3	2	4
Permethrin	0.01	1	4	0	1
	0.05	3	1	0	0
	0.1	4	3	0	1

Midges which were submitted to deltamethrin and survived showed a confluence of sensilla with a substance that aggregated them, as well as an abnormal rigidity and a central concavity. Those which were submitted to deltamethrin and died showed also an aggregation and an uncoordinated spatial orientation, as well as an altered anatomical aspect.

Midges submitted to permethrin that survived showed a confluence of structures, with production of a substance which aggregated them. Those who died after permethrin exposure showed sensilla basiconica with anatomical alterations, rigid and confluent, some of them with a central concavity.

5.5. Discussion

The response of sensilla basiconica to exposure of the two insecticides give them altered conformations (without a natural bulb-shaped form), probably due to their close or effective contact with the active ingredients. Since these morphological modifications were observed in dead and alive individuals but not in the control group, we observe that insecticide exposure induced physical changes in sensorial organs. The alterations observed in all *C. imicola* midges which were submitted to permethrin were the same observed in *C. imicola* that survived to deltamethrin exposition. The aggregation of sensilla with uncoordinated spatial orientation and altered anatomical aspect was only observed in specimens submitted to deltamethrin and that died after exposition. It is well known that deltamethrin is more efficient/toxic than permethrin (Soderlund *et al*., 2002; Soderlund, 2012; WHO, 2013) and, in

this way, this gradient of morphological alterations, from the most subtle to the most exacerbated, were expectable.

Besides the lethal effect of insecticides, these morphological modifications could alter the host seeking behaviour in individuals that survive to exposure and maybe reduce the number of bites and hence the transmission of pathogenic agents.

In the major part of the individuals submitted to the active ingredients, sensilla basiconica from both 3rd palpus segments were altered or completely destroyed, after 24h of exposure (80.85%). Sometimes, while sensilla basiconica from one of the 3rd palpus segment were affected, sensilla from the contralateral palpus were intact (19.15%), being this characteristic observed with a bigger frequency on specimens exposed to deltamethrin at the higher concentration (0.001%) (6 specimens out of 17).

The existence of non-affected sensilla may be due to a shorter time of exposure/contact with the active ingredient, probably being connected with the quick death of those specimens. However, the two individuals that survived after the exposition to the highest concentration of deltamethrin with functional sensilla basiconica in the contralateral palpus must be taken into account, since this situation suggests that some individuals resist to the application of insecticide, being able to find a host after exposition, as they still have their contralateral sensilla without morphological alterations. Although these midges have been captured in private farms with reduced use of insecticides on the animals or pesticides on corps, they could have been previously in regions with farms where the insecticide molecules are used intensively and possible show some degree of resistance to permethrin and/or deltamethrin.

Insecticidal pour-on products exert their effect in two-ways: primarily, they are highly toxic to insects landing on the treated animal, often killing them within minutes of their landing on the host; secondarily, they exert a contact irritation that may reduce the probability of the insect successfully initiating or completing a blood meal from the host (Venail *et al*., 2015). It is assumed that the lethal effect of pyrethroids is more important than the organophosphates (Venail, 2015).

With pour-on and spray formulations, permethrin (a 1st generation type I non α-cyano synthetic pyrethroid, providing less stability) give shelter against biting midges for at least 28-35 days (Schmahl *et al*., 2009b; Venail, 2015), killing a high number of *Culicoides* after only 15 seconds of exposure (Schmahl *et al*., 2009a). Venail (2015) reported that the diagnostic dose (twice the LC_{99}) of permethrin for field collected female *C. imicola* (Corsica, France) was 0.77%.

Deltamethrin (a 2nd generation type II α-cyano synthetic pyrethroid) interferes with sodium channels of *Culicoides* nerve axons that result in delayed repolarisation and paralysis (Casida, Gammon, Glickman & Lawrence, 1983). Some studies have shown that this insecticide (as well as other pyrethroids) used as pour-on application are rather confidential in quickly killing attacking females of different *Culicoides* species when they come into

contact with treated sheep wool or cattle hair (Mehlhorn *et al*., 2008a; Mehlhorn *et al*., 2008b; Schmahl *et al*., 2009a). In sheep, the efficacy of topic deltamethrin varies greatly, with effective *Culicoides* mortality occurring between 10 days to 5 weeks (Mullens *et al*., 2010; Venail *et al*., 2011; Weiher, Bauer, Mehlitz, Nijhof & Clausen, 2014). However, susceptibility to deltamethrin was found to be higher in *C. nubeculosus* colonies than in field populations of *C. obsoletus* or *C. imicola* (Venail *et al*., 2011), probably due to a higher exposure to insecticides than the other two species. *C. obsoletus* had a high susceptibility when submitted to deltamethrin at concentrations between 0.00105 and 0.00203% (Venail *et al*., 2011; Del Rio *et al*., 2014), suggesting that, although deltamethrin do not possess a repellent effect in horses (Robin *et al*. 2015), it seems unlikely that these biting midges gain resistance against this active ingredient. Venail (2015) reported that the diagnostic dose of deltamethrin for field collected female *C. imicola* (Corsica, France) was 0.007%.

Since the analysed specimens were not separated into two different populations (from France and Spain, respectively) and diagnostic doses have only been reported to specific populations (Venail, 2015), further studies must be performed to understand possible insecticide resistance in other populations from different areas or regions.

With the growing restrictions on the use of insecticides in the European continent, the list of active substance authorized in the veterinary context is short and will be higher in the forthcoming years (Venail, 2015). This means that in the medium and long term, the repeated use of one or two active substances will inevitably result in the appearance of resistance to these insecticides and there will be no other chemical control effective against vectors of diseases (including *Culicoides*) (Venail, 2015). This has already been observed at the horn flies in the US (Szalanski, Black & Broce, 1991; Oyarzún, Quiroz & Birkett, 2008; Oyarzún, Li & Figueroa, 2011), and in mosquitoes (*Aedes aegypti*) in the Caribbean (Dusfour *et al*., 2011; Marcombe *et al*., 2012), Central America (Bisset *et al*., 2013) and South America (Lima *et al*., 2011; Maciel-de-Freitas *et al*., 2014).

These results show that small concentrations of the referred active ingredients have a positive effect in both killing *Culicoides* midges and inducing anatomical alterations in sensorial organs and, in this way, small concentrations of both permethrin and deltamethrin must be taken into account when preventing *Culicoides* biting midges. Further investigations in different populations must be performed concerning other sensorial organs present in antennas or abdomen of biting midges to understand the impact of these active ingredients in different sensorial organs of *Culicoides* species and to comprehend the mechanisms behind insecticide resistance in biting midges.

Chapter 6: Conclusions and Future Perspectives

The present work allowed for the collection of data concerning *Culicoides* species captured in multiple farms from mainland Portugal, Azores and Madeira archipelagos during a ten year period, starting in 2005, after a BTD outbreak.

The evaluation of Portuguese *Culicoides* fauna showed their relative frequency, presence in some regions of Portugal and seasonality, revealing that most of the biting midges species can persist for 6 or more months in Portuguese territory, being capable of maintaining several generations per year and, probably, some arboviruses. Taking into account the high number of *Culicoides* species detected in Portugal for the first time and since some of them are vectors of VBD, a continuous entomological surveillance must be performed to avoid serious economic losses in case of an outbreak. In this context, further studies of vector competence and vector capacity concerning these species must be performed in order to understand their real role in VBD transmission.

This study mentions 22 *Culicoides* species for the first time in Portugal, including *C. dewulfi* and *C. montanus* in mainland Portugal, after a more detailed study performed with *Culicoides* species from Obsoletus group, all of them implicated in the transmission of several arboviruses to animals. Species from that group were also referred for the first time in Azores archipelago islands where they were never reported. Moreover, with exception of the Flores and Corvo islands, *C. circumscriptus* and *C. newsteadi* were also referred for the first time in Azores archipelago.

Furthermore, a description of *Culicoides paradoxalis*, a new species for science, was referred in this thesis. Since this species belongs to *Culicoides* subgenus, which possess, at least, one recognized vector of BTD, further studies must be performed in order to understand its role as a vector, together with other similar species already incriminated as potential vectors of BTD (*C. punctatus* and *C. newsteadi*).

As a result of this retrospective study on *Culicoides* species, an identification key for Portuguese female *Culicoides* was elaborated, which will contribute with important information concerning research and educational areas. This work also contributed to a better identification of species inside Obsoletus group by redefinition of preexistent intervals concerning two morphological structures (L/W ratio of the 3rd palpus segment and spermathecae size). Finally, several anatomical aberrations inside Obsoletus complex were observed and documented, some of them for the first time.

Further studies with specimens that possess intermediary characteristics between known *Culicoides* species must be performed in order to understand their actual taxonomic position.

A risk analysis was performed concerning *Culicoides* species vectors of VBD and those considered as potential vectors of VBD. This work showed that different edaphoclimatic variables have both positive and negative influence in different *Culicoides* species occurrence. After the evaluation of the different variables, presence/absence probability maps were constructed, with the respective cut-off values. Thus, this work suggests different

Culicoides species preferences and how different variables influence their occurrence in different seasons, contributing to a better understanding of these species dynamics and being an extremely important auxiliary information to further studies concerning ecological preferences and control measures studies that must be taken in order to reduce the risk of VBD and other diseases outbreaks.

Finally, evaluation of *C. imicola* morphological modifications in sensorial organs localized in the 3rd palpus segment, used for host detection, was performed after an assay with pyrethroid insecticides, showing that *C. imicola* female specimens have deep morphological alterations on those organs, probably leading to biting midges death due to a lack of host detection in order to perform their blood meal. Further research must be performed concerning other sensorial organs present in antennas or abdomen of biting midges to understand the impact of these active ingredients in different sensorial organs of *Culicoides* species and to understand how a continuous exposition to the same active ingredients may lead to resistances, like it happen in other Diptera.

Further work on *Culicoides* genus must be done concerning other possible hosts and feeding preferences. The degree of vector-host association is a key predictor of vector capacity and transmission of VBD. Thus, understanding host-feeding pattern of vector species populations and their variations in space and time is important since it can contribute to a better knowledge of their respective roles in pathogen transmission, and, consequently, the design of accurate vector control measures or strategies.

Finally, a deep research concerning both larval and adult biting midges breeding habitats must be performed in order to implement different control strategies for *Culicoides* population and, therefore, the transmission of VBD.

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Annexes

Annex 1.1. – First description of *Culicoides* species from Palearctic and other Earth ecozones by author(s) and year (Mathieu *et al*., 2012).

***Culicoides* species**	**Author(s), Year**	**Palearctic ecozone**	**Other ecozones**
C. achrayi	Kettle & Lawson, 1955	X	
C. alazanicus	Dzhafarov, 1961	X	
C. albihalteratus	Goetghebuer, 1935	X	
C. arboricola	Root & Hoffman, 1937		X
C. atripennis	Shevchenko, 1972	X	
C. begueti	Clastrier, 1957	X	
C. brevitarsis	Kieffer, 1917		X
C. brunnicans	Edwards, 1939	X	
C. cataneii	Clastrier, 1957	X	
C. chiopterus	(Meigen, 1830)	X	
C. circumscriptus	Kieffer, 1918	X	
C. clastrieri	Callot, Kremer & Déduit, 1962	X	
C. corsicus	Kremer, Leberre & Beaucournu-Saguez, 1971	X	
C. deltus	Edwards, 1939	X	
C. derisor	Callot & Kremer, 1965	X	
C. dewulfi	Goetghebuer, 1936	X	
C. fagineus	Edwards, 1939	X	
C. fascipennis	(Staeger, 1839)	X	
C. festivipennis	Kieffer, 1914	X	
C. furcillatus	Callot, Kremer & Paradis, 1962	X	
C. gejgelensis	Dzhafarov, 1964	X	
C. haranti	Rioux, Descous & Pech, 1959	X	
C. heliophilus	Edwards, 1921	X	
C. heteroclitus	Kremer & Callot, 1965	X	
C. imicola	Kieffer, 1913	X	
C. impunctatus	Goetghebuer, 1920	X	
C. indistinctus	Khalaf, 1961	X	
C. jumineri	Callot & Kremer, 1969	X	
C. jumineri near *C. bahrainensis*	Boorman, 1989	X	
C. jurensis	Callot, Kremer & Déduit, 1969	X	

Annex 1.1. – First description of *Culicoides* species from Palearctic and other Earth ecozones by author(s) and year (Mathieu et al., 2012) (Continuation).

***Culicoides* species**	**Author(s), Year**	**Palearctic ecozone**	**Other ecozones**
C. kibunensis	Tokunaga, 1937	X	
C. kingi	Austen, 1912	X	
C. kurensis	Dzhafarov, 1960	X	
C. longipennis	Khalaf, 1957	X	
C. lupicaris	Downes & Kettle, 1952	X	
C. malevillei	Kremer & Coluzzi, 1971	X	
C. maritimus	Kieffer, 1924	X	
C. montanus	Shakirzjanova, 1962	X	
C. newsteadi	Austen, 1921	X	
C. nubeculosus	Meigen, 1830	X	
C. nuntius	Cambournac, 1970	X	
C. obsoletus	(Meigen, 1818)	X	
C. odiatus	Austen, 1921	X	
C. pallidicornis	Kieffer, 1919	X	
C. paradoxalis	Ramilo & Delécolle, 2013	X	
C. parroti	Kieffer, 1922	X	
C. paolae	Boorman, 1996	X	
C. phlebotomus	Williston, 1896		X
C. pictipennis	(Staeger, 1839)	X	
C. picturatus	Kremer & Déduit, 1961	X	
C. pseudopallidus	Khalaf, 1961	X	
C. pulicaris	(Linnaeus, 1758)	X	
C. punctatus	(Meigen, 1804)	X	
C. puncticollis	(Becker, 1903)	X	
C. remmi	Damian-Georgescu, 1972	X	
C. riebi	Delécolle, Mathieu & Baldet, 2005	X	
C. riethi	Kieffer, 1914	X	
C. rochenus	Cambournac, 1970	X	
C. sahariensis	Kieffer, 1923	X	
C. santonicus	Callot, Kremer, Rault & Bach, 1966	X	
C. scoticus	Downes & Kettle, 1952	X	

Annex 1.1. – First description of *Culicoides* species from Palearctic and other Earth ecozones by author(s) and year (Mathieu et al., 2012) (Continuation).

***Culicoides* species**	**Author(s), Year**	**Palearctic ecozone**	**Other ecozones**
C. semimaculatus	Clastrier, 1958	X	
C. shaklawensis	Khalaf, 1957	X	
C. simulator	Edwards, 1939	X	
C. sonorensis	Wirth & Jones, 1957		X
C. stigma	(Meigen, 1818)	X	
C. subfagineus	Delécolle & Ortega, 1998	X	
C. subfasciipenis	Kieffer, 1919	X	
C. tbilisicus	Dzhafarov, 1964	X	
C. univittatus	Vimmer, 1932	X	
C. vexans	(Staeger, 1839)	X	

Annex 1.2. – Synonymies of *Culicoides* species referred in Portugal (1952-2005).

***Culicoides* species**	**Synonymies**[1]
C. albihalteratus[2]	*C. pseudoheliophilus*[2]
C. circumscriptus	*C. albosignatus, C. algarum, C. edwardsi, C. kirovabadicus, C. matsuensis, C. nadayanus, C. pictidorsum, C. polymaculatus, C. pulcher, C. salicola, C. albonotatus, C. meridionalis*
C. fascipennis	*C. turficola, C. albonotatus, C. distictus*
C. festivipennis	*C. odibilis, C. winnertzi*
C. imicola	*C. minutus, C. pseudoturgidus, C. iraqensis, C. pallidipennis*
C. impunctatus	*C. minor*
C. indistinctus	*C. odiatus, C. lailae, C. niger, C. conicus, C. kurektshaicus*
C. kibunensis	*C. sitinohensis, C. cubitalis, C. ponkikiri*
C. longipennis	*C. flavisimilis*
C. maritimus	*C. submaritimus*
C. newsteadi	*C. edwardsi, C. halophilus, C. biclavatus, C. edwardsianus*
C. nubeculosus	*C. puncticollis, C. punctaticollis*
C. obsoletus	*C. concitus, C. heterocerus, C. intermedius, C. kabyliensis, C. lacteinervis, C. pegobius, C. rivicola, C. seimi, C. sintrensis, C. varius, C. yezoensis, C. clavatus, C. obscuripes*
C. odiatus	*C. indistinctus, C. lailae, C. niger, C. conicus, C. kurektshaicus*
C. pallidicornis	*C. brunneiscutellatus, C. niger, C. susae, C. bruneoscutellatus, C. dileucus*
C. pulicaris	*C. flaviplumus, C. pullatus, C. sawamotoi, C. setosinervis, C. stephensi, C. cinerellus, C. quinquepunctatus*
C. punctatus	*C. kasachstanicus, C. ocellaris*
C. puncticollis	*C. bipunctatus, C. distigma, C. donatieni, C. flavitarsis, C. griseovittatus, C. luteosignatus, C. sciniphes, C. tripunctatus, C. vavrai, C. wenigi, C. algecirensis, C. impressus*
C. sahariensis	*C. baghdadensis, C. coluzzii, C. similis-baghdadensis*
C. subfasciipennis	*C. analis*
C. tbilisicus[2]	*C. dendriticus*[2], *C. ribeiroi*
C. univittatus	*C. agathensis*
C. vexans	*C. pungens, C. ajbassovi, C. perpungens*

[1]Fauna Europaea (2015); [2]Both designations are valid as species names (Mathieu *et al*., 2012; Fauna Europaea, 2015).

Annex 1.3. – Host preferences of different *Culicoides* species from Palearctic ecozone.

Subgenus	Species	Mammalian species	Avian species	N.º of hosts	References[1]
Avaritia	*C. chiopterus*	*Bos taurus* *Capra hircus* *Capreolus capreolus* *Ovis aries* *Equus caballus* *Homo sapiens*	*Columba palumbus*	7	A-F
	C. dewulfi	*Bos taurus* *Ovis aries* *Sus scorfa*[2] *Oryctolagus cuniculus* *Equus caballus* *Homo sapiens*	-	6	A,B,D-G
	C. imicola	*Bos taurus* *Ovis aries* *Equus caballus*	-	3	L
	C. obsoletus	*Bos taurus* *Capra hircus* *Capreolus capreolus* *Cervus elaphus* *Ovis aries* *Oryctolagus cuniculus* *Equus caballus* *Homo sapiens* *Mus musculus*	*Anas platyrhynchos* *Columba palumbus*	11	A-I
	C. scoticus	*Bos taurus* *Capra hircus* *Capreolus capreolus* *Ovis aries* *Sus scorfa*[2] *Oryctolagus cuniculus* *Equus caballus* *Homo sapiens*	*Anas platyrhynchos* *Columba palumbus*	10	A-H

Annex 1.3. – Host preferences of different Culicoides species from Palearctic ecozone (Continuation).

Subgenus	Species	Mammalian species	Avian species	N.º of hosts	References[1]
Beltranmyia	*C. circumscriptus*	*Homo sapiens* *Equus caballus* Buffaloes	*Columba palumbus* *Corvus corone* *Phylloscopus trochilus* *Pica pica* *Turdus merula* *Turdus philomelos* *Asio otus*	10	B,E,J,Q
Culicoides	*C. deltus*	*Bos taurus* *Homo sapiens*	-	2	C,D,G
	C. impunctatus	*Bos taurus* *Ovis aries* *Equus caballus* *Homo sapiens* Deer	-	5	B,P
	C. lupicaris	*Bos taurus* *Ovis aries* *Sus scorfa*[2] *Oryctolagus cuniculus* *Equus caballus*	-	5	A,B,E,F
	C. newsteadi	*Bos taurus* *Ovis aries* *Homo sapiens*	-	3	A,B,G,K
	C. pulicaris	*Bos taurus* *Capra hircus* *Cervus elaphus* *Oryctolagus cuniculus* *Ovis aries* *Equus caballus* *Homo sapiens* Buffaloes	*Gallus gallus*	9	A-F,K,M,V

Annex 1.3. – Host preferences of different Culicoides species from Palearctic ecozone (Continuation).

Subgenus	Species	Mammalian species	Avian species	N.º of hosts	References[1]
Culicoides	*C. punctatus*	*Bos taurus* *Capra hircus* *Capreolus capreolus* *Cervus elaphus* *Ovis aries* *Alces alces* *Oryctolagus cuniculus* *Equus caballus* *Homo sapiens* *Microtus savii*	*Anas platyrhynchos* *Columba palumbus* *Gallus gallus* *Luscinia svecica*	14	A-C,E,F,J,K
Monoculicoides	*C. nubeculosus*	*Ovis aries* *Equus caballus* *Homo sapiens*	-	3	R
	C. parroti	*Bos taurus* *Ovis aries* *Equus caballus* Deer	-	4	K,N,O,S
	C. puncticollis	*Bos taurus* *Equus caballus* *Homo sapiens*	-	3	Q,W
Oecacta	*C. clastrieri*	*Homo sapiens*	*Tadorna ferruginea* *Turdus philomelos*	3	D,G
	C. gejgelensis	*Equus caballus* *Homo sapiens*	-	2	Q
	C. kibunensis	*Bos taurus* *Equus caballus* *Homo sapiens*	*Columba palumbus* *Cyanistes caerulescens* *Acrocephalus palustris* *Emberiza citrinella* *Erithacus rubecula* *Sylvia atricapilla*	9	D,E,G,Q,T
	C. semimaculatus	*Homo sapiens*	*Erithacus rubecula*	2	D,G
	C. simulator	-	*Cyanistes caerulescens*	1	T

Annex 1.3. – Host preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Mammalian species	Avian species	N.º of hosts	References[1]
Oecacta	*C. alazanicus*	*Homo sapiens*	*Columba palumbus* *Anthus trivialis* *Delichon urbica* *Luscinia luscinia* *Muscicapa striata* *Oriolus oriolus* *Parus major* *Phylloscopus trochilus* *Pica pica* *Sylvia borin* *Turdus merula* *Turdus philomelos* *Ardea purpurea* *Ixobrychus minutus* *Asio otus*	16	J
	C. festivipennis	*Bos taurus* *Ovis aries* *Homo sapiens*	*Columba palumbus* *Streptopelia decaocto* *Anthus trivialis* *Oriolus oriolus* *Passer domesticus* *Passer montanus* *Passer hispaniolensis* *Pica pica* *Ardea purpurea* *Nycticorax nycticorax* *Asio otus*	14	B,D,E,G,J,K,X
	C. vexans	*Bos taurus* *Capreolus capreolus* *Homo sapiens*	-	3	E

Annex 1.3. – Host preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Mammalian species	Avian species	N.º of hosts	References[1]
Silvaticulicoides	*C. achrayi*	*Bos taurus* *Oryctolagus cuniculus*	-	2	B,F,M
	C. pallidicornis	*Bos taurus* *Capra hircus* *Equus caballus* *Oryctolagus cuniculus* *Homo sapiens*	-	4	D-G,Q
	C. picturatus	*Bos taurus* *Oryctolagus cuniculus*	-	2	F,M,U
	C. subfasciipennis	*Homo sapiens*	-	1	Q

[1]A: Garros *et al*., 2011; B: Pettersson *et al*., 2013; C: Lassen *et al*., 2011; D: Santiago-Alarcon *et al*., 2012; E: Lassen *et al*., 2012; F: Ninio *et al*., 2011b; G: Santiago-Alarcon *et al*., 2013; H: Viennet *et al*., 2011; I: Martínez-de la Puente *et al*., 2012; J: Boveba *et al*., 2014; K: Calvo *et al*., 2012; L: Logan *et al*., 2010; M: Ninio *et al*., 2011a; N: Mellor *et al*., 1974b; O: Gerry *et al*., 2009; P: Mands *et al*., 2004; Q: Dzhafarov, 1964b; R: de Heredia & Lafuente, 2011; S: Kremer, 1964; T: De la Puente *et al*., 2009; U: Ninio *et al*., 2010; V: Boorman, 1989; W: Braverman *et al*., 1974; X: Kitaoka & Morii, 1964; [2]*Sus scorfa* includes wild boars and domestic pigs.

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone.

Subgenus	Species	Favourable habitats	Non-favourable habitats	References[1]
Avaritia	*C. chiopterus*	Cattle and sheep dung in pastures (preferably near forests or shaded areas) Horse dung Old heaps of manure Nutrient enriched soil Woodlands Larvae in mushrooms, elm trunks and marshes with rotting vegetation Marshy lands with rotting vegetation	Soil with low organic matter fraction Waterlogged soils Dried dung	E,F,O-R,U, W-AB AK,AS,AW
	C. dewulfi	Cattle dung in pastures near forests (shaded areas) Heaps of sheep dung and straw Horse dung Old heaps of manure Molehill soil Woodlands Marshy habitats	Moisture-retentive, nutrient enriched soil Waterlogged soils Dried dung	F,O-R,U W-AB AS,AV,AW
	C. imicola	Sparsely vegetated areas in full sunlight Open areas with mud and partially contaminated with cattle dung Water-soil interface on the banks of ponds, streams and other water sources with a high content of organic matter	Annual rainfall >1000mm Water run-off due to terrain slope and soil texture Rapid soil surface layer desiccation	A,H,AC AT-AV
	C. scoticus	Forest leave litter Dried dung in cowshed dungs Maize silages Rotting fungi Mud ruts Marshy habitats	Arable areas Manure Soils with high levels of calcium and magnesium	A-D,P-T,V

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Favourable habitats	Non-favourable habitats	References[1]
Avaritia	*C. obsoletus*	Broad woodland leave litter and vegetation Dried dung in cowshed walls Maize silages Unsaturated, anthropogenic soils in shaded areas Compost heaps of trees Tree holes and rotting trunks of banana trees Marginal vegetation in open water Faecal contaminated straw Different types of manure piles (horses and cows) Masses of corn stover Marshy habitats Tanks with stagnant water	Arable areas Waterlogged soils Soils with high levels of calcium and magnesium	A-R V,AP,AV
Beltranmyia	*C. circumscriptus*	Pond edge mud Substrates near water pools Muds enriched with organic matter Moist soils Faecal contaminated puddles Mud along pond shoreline Broad woodland leaf litter Bare mud without vegetation Near halophilus vegetation, salt margins and wetlands flooded by tides Open areas with high luminosity, rich in organic matter, low pH and electric conductivity	-	F,H,K,AM,AN, AS,AV,AW
Culicoides	*C. fagineus*	Mud and decaying matter in tree holes Larvae found in wet places, close to tree roots and elm sap	-	P, AJ-AL,AV
	C. impunctatus	Marshes Swamp soils Reed grasses Peat-rich areas	-	AG-AI,AW
	C. lupicaris	Swamp mud with little vegetation	-	AW

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Favourable habitats	References[1]
Culicoides	*C. newsteadi*	Muds near pools and ponds	AV,AW
	C. punctatus	Fallen leaves River edges Forest mud Wet grazed field with manure Waterlogged soils Marshy areas Soil with algae Stagnant water Wet soils between silages reserves Pond slit Swamps (with water level above the soil surface)	F,R,AD-AF,AS
	C. pulicaris	River edges Forest mud Wet grazed field with manure Waterlogged soils near lakes, bogs and marshy places Molehill soil Ponds Forest leaf litters Pond slit Swamps (with water level above the soil surface)	F,P,W, AD-AF, AO,AP
Monoculicoides	*C. nubeculosus*	Soil with algae Stagnant water Pond slit Mud contaminated with cattle, sheep, chickens, ducks and geese faeces. Marginal vegetation near open water Mud along pond shoreline Broad woodland leaf litter Bare mud without vegetation Mud with mean salinity and neutral pH	F,P,Q,AO-AQ,AS,AX

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Favourable habitats	References[1]
Monoculicoides	*C. parroti*	Floating algae Faecal contaminated mud Woodlands Wet soil and clay near rivers and reeds	AJ,AK,AN,AV
	C. puncticollis	Deserts and regions with a hot and dry climate Faecal contaminated mud near water reservoirs Moist soil with organic matter	H,AK,AM
Oecacta	*C. alazanicus*	Larvae found in clay along a channel	AW
	C. albihalteratus	Undergrowth in wetlands	P
	C. begueti	Soil, water and rotten vegetation collected from oak and elm holes	H,P
	C. cataneii	Muds and organic matter Clay from ponds Wet grass next to a cattle farm	H,AV,AR
	C. clastrieri	Mud near water collections	AW
	C. corsicus	Muds	AV
	C. festivipennis	Pond edge mud Substrates near water pools Muds enriched with organic matter Moist soils Soil with algae Stagnant water Pond slit Mud along pond shoreline Broad woodland leaf litter Bare mud without vegetation Swamps (with water level above the soil surface) Preference for habitats with a high pH and high concentrations of phosphorus, potassium and zinc	F,H,P-R AM,AN,AR,AS,AW

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

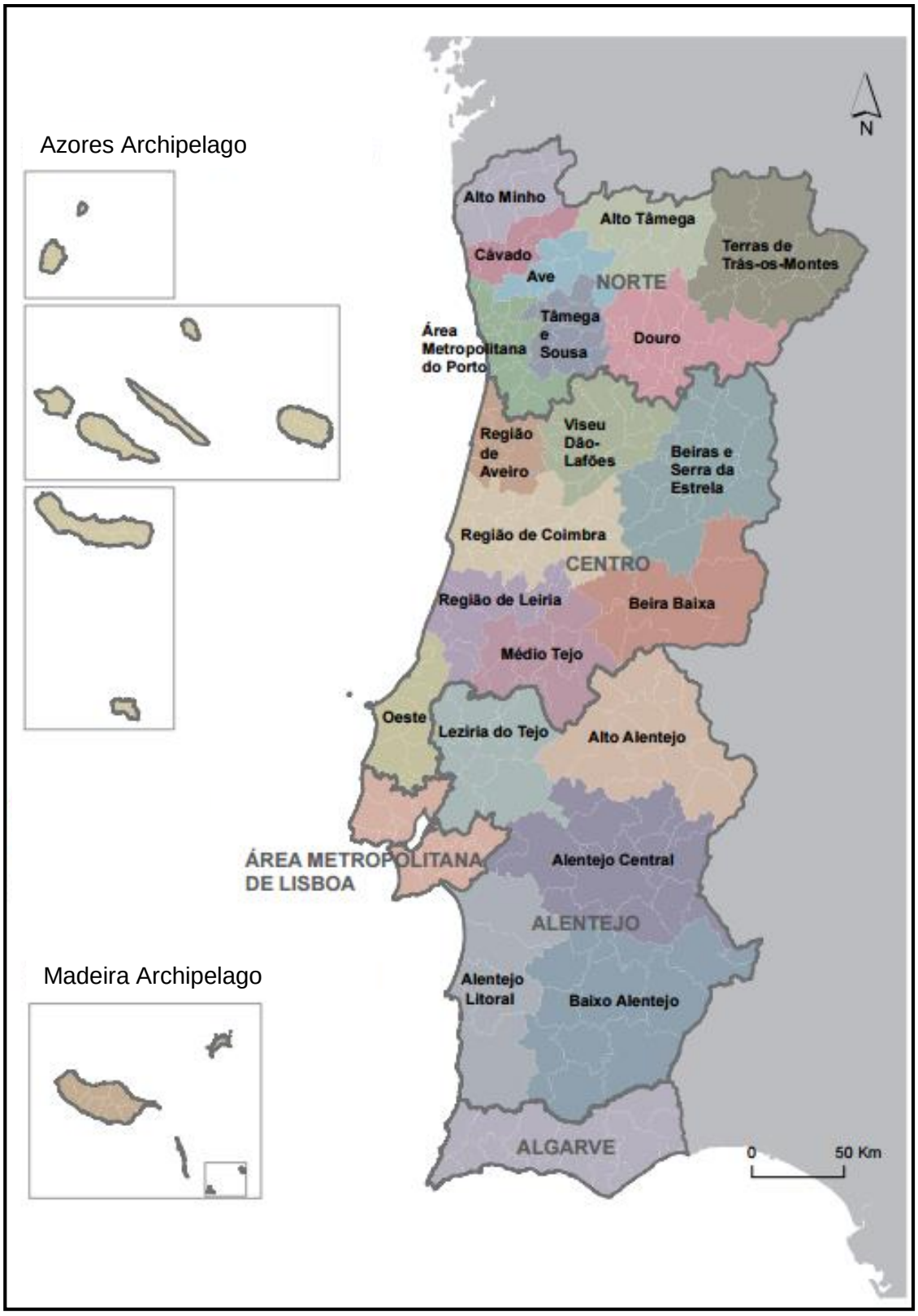
Subgenus	Species	Favourable habitats	References[1]
Oecacta	*C. gejgelensis*	Mud margins of rivers and marshes with low or high organic matter Moist soil with organic matter Moist soils Dripping water Sewage channels Reed sites Garden watering channels Watering channels Rain pools Sites along the stream	AK,AM,AN,AX
	C. haranti	Arboreal species like *C. begueti*	P
	C. heteroclitus	Muds with or without dung	AV
	C. indistunctus	Muds	AV
	C. kibunensis	Eutrophic fresh water marshes Larvae are found in mud with decaying vegetation in forests Clay near grass and pond	F,AR,AV,AW
	C. kurensis	Muds	AV
	C. longipennis	Larvae found in areas nearby mud contaminated with feces near water reservoirs and soils withtout organic matter	AN,AV
	C. maritimus	Salt marshes Brackish water puddles Mud rich in organic matters near water reservoirs Dripping water and watering channels	F,AK,AM,AV
	C. odiatus	Mud rich in organic matters near the water reservoirs Reed sites Watering channels Rain pools Mud near dams	AM,AV

Annex 1.4. – Habitat preferences of different *Culicoides* species from Palearctic ecozone. (Continuation).

Subgenus	Species	Favourable habitats	References[1]
Oecacta	*C. pseudopallidus*	Muds	AV
	C. sahariensis	Muds	AV
	C. simulator	Forests Larva found in clay from waterlogged hayfields	F,AW
	C. univittatus	Muds Adults found next to a pond Specimens found in clay	AR,AV
	C. vexans	Muds Larva found in clay from waterlogged hayfields in shadowed areas Adults breed in moist soil under shrubs and trees	AV,AW,AZ
Silvaticulicoides	*C. achrayi*	Mud from marshy areas Mud contaminated with cattle dung Forest and woodland habitats First larval stages in decaying matter	P,AV,AW
	C. fascipennis	Mud collected between vegetation in open areas Decaying vegetable matter	F,BA
	C. pallidicornis	Connected to forest areas Decaying matter Marshy areas	F,AW
	C. picturatus	Non-saline wetland habitats	AW
	C. subfasciipennis	Mud rich in organic matters near water reservoirs Wet woodlands	AM,AV,AW

[1]A: Conte *et al*., 2007; B: Zimmer *et al*., 2010; C: Ninio *et al*., 2011a; D: Zimmer *et al*., 2008; E: Hill, 1947; F: Kettle *et al*., 1952; G: Weinburgh *et al*., 1962; H: Braverman *et al*., 1974; I: Trukhan, 1975; J: Mirzaeva *et al*., 1976; K: Mellor *et al*., 1979; L: Chaker, 1983; M: Mathieu, 2005; N: Zimmer *et al*., 2012; O: Murray, 1957; P: Kremer, 1965; Q: Harrup *et al*., 2013; R: González *et al*., 2013; S: Buxton, 1960; T: Boorman *et al*., 1970; U: Kettle, 1962a; V: Zimmer *et al*., 2013a; W: Zimmer *et al*., 2014; X: Glushchenko *et al*., 2008; Y: Scolamacchia *et al*., 2014; Z: Lüken *et al*., 2014; AA: Bishop *et al*., 2005b; AB: Cannon *et al*., 1966; AC: Wittmann *et al*., 2001; AD: Kirkeby *et al*., 2009; AE: Nevill *et al*., 2007; AF: EFSA, 2007; AG: Kettle, 1990; AH: Blackwell *et al*., 1994; AI: Carpenter *et al*., 2001; AJ: Edwards, 1939; AK: Dzhafarov, 1964; AL: Sánchez-Covisa *et al.*, 1979; AM: Uslu *et al*., 2007; AN: Uslu *et al*., 2010; AO: Konurbayev, 1965; AP: Dzhafarov, 1976; AQ: Downes, 1950; AR: Foxi *et al*., 2010; AS: Zimmer *et al*., 2013b; AT: Walker, 1977; AU: Braverman, 1978; AV: Pena, 2003; AW: de Heredia & Lafuente, 2011; AX: Uslu, 2003; AZ: Jobing, 1953; BA: Glukhova, 1979.

Annex 2.1. – The nomenclature of territorial units for statistics, subdivision 3 (available in: http://economiafinancas.com/2015/conheca-a-nova-nomenclatura-das-unidades-territoriais-para-fins-estatisticos-nuts-2013/, accessed on the 28th October, 2015).



Annex 2.2. – Hoyer's Medium (adapted from de Heredia & Lafuente, 2011).

Material:

2 Beaker glasses of 500 ml each

Glass rod

Hot plate

30 gauzes

Funnel

Reagents (for a final volume of 300 ml aprox.):

100 ml of distilled water

60 g of gum arabic (crystals)

400 g of chloral hydrate

40 ml of glycerol

Procedure:

1 – Mix gum arabic with distilled water in a Beaker glass and take it to a hot plate until it boils, stirring very well to dissolve as much as possible.

2 – After dissolution of the gum arabic, add chloral hydrate and stir very well until it dissolves completely.

3 – Allow the solution to cool slightly and add glycerine, stirring very well.

4 – Filter the solution with a funnel into another Beaker glass, using 8 to 10 overlapping gauzes, in order to create enough porosity to allow the passage of Hoyer's medium and retain the impurities at the same time. Repeat this step 3 times.

5 – To examine if Hoyer's medium has low or no impurities at all, put a drop between a slide and a coverslide and observe it under the microscope.

Annex 2.3. – Synonyms of *Culicoides* species referred for the first time in mainland Portugal during the NESP (2005-2013) and VectorNet European network (2015).

***Culicoides* species**	**Synonymies**[1]
C. alazanicus	*C. musilator*
C. deltus	*C. lupicaris*[2]
C. heliophilus	*C. kobachidzei, C. latifrontis*
C. kingi	*C. nilotes*
C. grisescens	*C. arschanicus*, *C. remmi*[3]
C. semimaculatus	*C. karajevi*

[1]Fauna Europaea (2015); [2]According to Fauna Europaea (2015), *C. lupicaris* is a synonym of *C. deltus*. The classification of Mathieu *et al*., (2012) was followed (*C. lupicaris* and *C. deltus* are two independent species); [3]According to Fauna Europaea (2015), *C. remmi* is a synonym of *C. grisescens*. The classification made by Mathieu *et al*., (2012) was followed (*C. grisescens* and *C. remmi* are two independent species).

Annex 4.1. – Meteorological data obtained from the closest meteorological stations to the farms.

***Culicoides* species**	**Temperatures (°C)[1]**			**Wind Speed (Km/h)[1]**			**Relative Humidity (%)[3]**		
	Min	**Mean[2]**	**Max**	**Min**	**Mean[2]**	**Max**	**Min**	**Mean[2]**	**Max**
C. achrayi	4.5	19.35	36.4	0	8.4	33.8	11	50.14	100
C. alazanicus	6.4	18.07	32.3	5	14.44	28.1	38	70.74	100
C. albihalteratus	6.4	17.13	29.8	4	11.03	30.2	19	59.63	98
C. begueti	10.2	23.29	40.4	3.2	10.82	21.2	18	46.73	88
C. cataneii	1.9	14.04	30.1	3.1	11.14	25.6	23	67.97	97
C. circumscriptus	2.6	19.50	40.4	0	11.69	39.6	9	58.34	100
C. clastrieri	6.1	20.98	39.2	5	10.69	24.1	10	48.73	100
C. corsicus	8	20.30	34.2	2.9	9.45	11.5	13	52.14	100
C. deltus	9.4	20.24	34.9	5	8.04	17.6	18	48.81	100
C. derisor	12.1	24.00	40.4	3.2	10.94	22.3	11	43.43	100
C. dewulfi	9.6	19.28	32.9	4.7	7.32	11.5	18	63.63	100
C. fascipennis	4.9	20.07	37.8	3.6	9.06	23.5	11	52.74	100
C. festivipennis	1.5	19.93	40.4	0	10.82	32.8	9	52.62	100
C. gejgelensis	-1.1	16.47	36.1	1.4	12.38	39.6	11	63.04	100
C. haranti	9.8	22.57	34	5.8	12.57	19.8	16	44.25	88
C. heliophilus	4.7	17.61	33	0	7.96	31.7	28	55.2	93
C. heteroclitus	6	20.85	40.4	2.5	10.64	20.9	9	50.07	100
C. imicola	-1.5	18.37	40.4	0	11.55	50.4	9	58.58	100
C. impunctatus	4.9	17.37	34.4	3.2	8.35	23.4	21	63.67	96
C. indistinctus	1.3	22.04	37.3	0	10.01	28.8	10	47.12	99
C. jumineri	4.2	17.78	34.6	0	10.44	16.6	11	58.18	94
C. jumineri near *C. bahrainensis*	4.1	20.81	40.4	2.5	11.52	24.5	9	51.96	100
C. jurensis	8.3	22.11	37.1	3.2	12.75	28.1	16	50.98	97
C. kibunensis	8.4	21.77	36.4	0	10.22	21.2	13	53.14	100
C. kurensis	5.5	22.16	40.4	4	11.33	22.3	9	47.78	100
C. longipennis	6	22.83	40.4	2.9	12.14	28.1	9	46.48	100
C. lupicaris	4.4	17.59	34.9	3.2	7.85	15.1	13	55.08	96
C. malevillei	11.7	21.37	34.2	7.6	13.8	21.2	16	50.67	100
C. maritimus	3.4	22.01	38.7	2.2	11.61	22.3	10	51.27	97
C. montanus	-2.8	17.46	39.2	0	10.35	33.8	10	62.90	100
C. newsteadi	-3.8	17.3	40.4	0	11.38	50.4	9	62.06	100
Nubeculosus group	4.1	20.73	40.4	3.6	12.35	26.3	9	50.34	100
C. obsoletus	-1.7	17.33	39.2	0	10.40	33.8	10	62.99	100
C. odiatus	7.3	21.75	36.5	1.4	11.74	50.4	9	53.87	100
C. pallidicornis	7.3	21.69	36.4	4.3	9.52	31.7	13	49.75	98

Annex 4.1. – Meteorological data obtained from the closest meteorological stations to the farms. (Continuation).

***Culicoides* species**	**Temperatures (°C)[1]**			**Wind Speed (Km/h)[1]**			**Relative Humidity (%)[3]**		
	Min	**Mean[2]**	**Max**	**Min**	**Mean[2]**	**Max**	**Min**	**Mean[2]**	**Max**
C. paradoxalis	5.2	19.74	36.5	5	13.35	28.1	10	61.05	99
Parroti group	7.8	20.77	33	0	8.83	14.4	11	51.46	93
C. picturatus	6.3	19.8	36.4	0	8.92	25.6	13	52.52	98
C. pseudopallidus	4.2	21.12	38.4	2.9	11.94	31.7	9	51.20	100
C. pulicaris	-3.1	17.67	39.2	1.4	9.05	41	9	62.68	100
C. punctatus	-2.6	17.49	40.4	0	10.54	41	9	59.42	100
C. remmi	11.2	20.35	28.5	5.8	8.93	11.9	26	50.5	83
C. riebi	14.5	23.58	33	4.7	8.47	11.5	19	46.33	75
C. sahariensis	8.3	23.06	40.4	6.7	12.43	23	9	48.67	100
C. santonicus	2.7	15.63	32.7	0	10.66	28.4	9	58.42	100
C. scoticus	0.1	16.52	38.6	0	9.70	33.8	11	66.01	100
C. semimaculatus	13.8	24.77	40.4	9	13.56	23	11	41	79
C. simulator	10.1	16.3	22.5	11.2	12.45	13.7	45	71	97
C. subfagineus	6.4	20.85	40.4	0	12.06	30.2	9	53.31	100
C. subfasciipennis	-3.8	21.2	38.8	2.5	10.21	19.8	12	50.75	100
C. tbilisicus	9	22.93	36.4	2.9	8.31	13.7	12	44.41	87
C. univittatus	-1.4	14.84	40.4	0	11.85	39.6	10	66.72	100
C. vexans	6.4	16.08	27.6	3.2	6.92	13.7	17	57.13	100

[1]Minimum values were registered in the night of capture and maximum values in the day of trap placement; [2]Mean values were obtained from all minimum and maximum values registered in each species captures; [3]Relative humidity was registered at 3 pm on the day of trap placement and at 9 am on the day of trap removal.