EL GÉNERO *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) EN EL PAÍS VASCO, NORTE DE ESPAÑA

THE GENUS *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) IN THE BASQUE COUNTRY, NORTHERN SPAIN

Memoria presentada por Mikel A. González González de Heredia **para optar al título de Doctor en Ciencias Biológicas**

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Dedicado
a mis padres

Miguel y Fer

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PUBLICATIONS

- **PUBLICATIONS LINKED WITH THE DOCTORAL THESIS**

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- **PUBLICATIONS OF CERATOPOGONIDAE NOT LINKED WITH THE DOCTORAL THESIS**

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C. achrayi Kettle and Lawson, 1955
C. alazanicus Dzhafarov, 1961
C. albicans (Winnertz, 1852)
C. almeidae Cambournac, 1961
C. barbosai Wirth and Blanton, 1956
C. bolitinos Meiswinkel, 1989
C. brevitarsis Kieffer, 1917
C. brunnicans Edwards, 1939
C. nujiangensis Liu, 1990
C. cataneii Clastrier, 1957
C. chiopterus (Meigen, 1830)
C. circumscriptus Kieffer, 1918
C. clastrieri Callot, Kremer and Deduit, 1962
C. comosioculatus Tokunaga, 1956
C. deltus Edwards, 1939
C. dewulfi Goetghebuer, 1936
C. dobyi Callot and Kremer, 1969
C. duddingstoni Kettle and Lawson, 1955
C. fagineus Edwards, 1939
C. fascipennis (Staeger, 1839)
C. festivipennis Kieffer, 1914
C. flavipulicaris Dzhafarov, 1964
C. floridensis Beck, 1951
C. furcillatus Callot, Kremer and Paradis, 1962
C. furens (Poey, 1853)
C. gejgelensis Dzhafarov, 1964
C. griseidorsum Kieffer, 1818
C. grisescens Edwards, 1939
C. heliophilus Edwards, 1921
C. hollensis (Melanter and Brues, 1913)
C. imicola Kieffer, 1913
C. immaculatus Lee and Reye, 1953
C. impunctatus Goetghebuer, 1920
C. kibunensis Tokunaga, 1937
C. kurensis cf. Dzharafov, 1960
C. longipennis Khalaf, 1957
C. lupicaris Downes and Kettle, 1952
C. maculatus (Shiraki, 1913)
C. maritimus Kieffer, 1924
C. melleus (Coquillett, 1901)
C. minutissimus (Zetterstedt, 1855)
C. mississippiensis Hoffman, 1926
C. molestus (Skuse, 1889)
C. montanus Shkirzjanova, 1962
C. newsteadi Austen, 1921
C. nubeculosus (Meigen, 1830)

C. nujiangensis Liu, 1990
C. obsoletus (Meigen, 1818)
C. odiatus Austen, 1921
C. ornatus Taylor, 1913
C. oxystoma Kieffer, 1910
C. pallidicornis Kieffer, 1919
C. paradisionensis Boorman, 1988
C. paraensis (Goeldi, 1905)
C. parroti Kieffer, 1922
C. pictipennis (Staeger, 1839)
C. picturatus Kremer and Deduit, 1961
C. poperinghensis Goetghebuer, 1953
C. pseudoheliophilus Callot and Kremer, 1961
C. pulicaris (Linnaeus, 1758)
C. punctatus (Meigen, 1804)
C. puncticollis (Becker, 1903)
C. reconditus Campbell and Pelham-Clinton, 1960
C. riethi Kieffer, 1914
C. salinarius Kieffer, 1914
C. sanguisuga (Coquillett, 1901)
C. santonicus Callot, Kremer, Rault and Bach, 1966
C. scoticus Downes and Kettle, 1952
C. segnis Campbell and Pelham-Clinton, 1960
C. semimaculatus Clastrier, 1958
C. shaklawensis Khalaf, 1957
C. simulator Edwards, 1939
C. sinanoensis Tokunaga, 1937
C. stigma (Meigen, 1818)
C. subfagineus Delécolle and Ortega, 1998
C. subfasciipennis Kieffer, 1919
C. tauricus Gutsevich, 1959
C. tissoti Wirth and Blanton, 1966
C. truncorum Edwards, 1939
C. univitattus Vimmer, 1932
C. utahensis Fox, 1946
C. variipennis (Coquillett, 1901)
C. vexans Staeger, 1839

Nomenclature according to Art Borkent in
"World Species of Biting Midges (Diptera: Ceratopogonidae)"

OBJECTIVES

In the present study, several biological and ecological aspects of the biting midges of genus *Culicoides* (Diptera: Ceratopogonidae) are investigated. The aim of the present doctoral thesis is to disseminate new insights about this genus throughout the Basque Country. The information presented here could be useful to improve the knowledge concerning this taxonomic group and enable us to be better prepared to prevent future outbreaks.

The specific objectives are as follows:

Chapter 1: THE GENUS *CULICOIDES* IN THE BASQUE COUNTRY: AN OVERVIEW OF *CULICOIDES* DIVERSITY

- Report the species diversity of genus *Culicoides* in the Basque Country region
- Drawing up of an illustrated taxonomic key for *Culicoides* species (females and males) inhabiting the Basque Country
- Description of species previously not found in the studied area

Chapter 2: MONITORING OF *CULICOIDES* MIDGES AFTER BTV OUTBREAKS, IN SHEEP FARMS AND NATURAL HABITATS FROM THE BASQUE COUNTRY

- Species composition of *Culicoides* spp. associated with sheep farms and natural wildlife habitats
- Diversity and abundance of *Culicoides* spp. in farms and natural habitats
- Phenology and population dynamics of *Culicoides* spp. in farms and natural habitats
- Spatial distribution of *Culicoides* spp. in farms and natural habitats
- Adult behaviour of *Culicoides* spp. (endophily and exophily)
- Detection of the presence and establishment of non-native potential vector species of the BTV in the Basque Country, such as *Culicoides imicola*

Chapter 3: A SURVEY OF *CULICOIDES* DEVELOPMENTAL SITES ON A FARM IN NORTHERN SPAIN, WITH A BRIEF REVIEW OF IMMATURE HABITATS OF EUROPEAN AND NEARCTIC SPECIES

- Determination of larval development sites of *Culicoides* on a farm and its surroundings in the Basque Country
- Phenology and emergence sites of *Culicoides* species with and without veterinary importance
- Study of the spatial and temporal distribution of *Culicoides* spp. adults.
- Influence of the humidity gradient and the vertical distribution on immature stages allocation
- Relationship between the light trap captures and the adult soil emergences
- Establishment of vector control strategies against immature stages of *Culicoides*

Chapter 4: LABORATORY AND FIELD EVALUATION OF CHEMICAL AND PLANT-DERIVED POTENTIAL REPELLENTS AGAINST *CULICOIDES* MIDGES IN THE BASQUE COUNTRY

- Evaluation and effect of the repellent activity of several putative repellents against *Culicoides* spp.
- Evaluation of the atractive activity of several putative attractants for *Culicoides* spp. (Appendix III).
- Evaluation in the field of the most promising repellents according to previous laboratory trials
- Comparison of the activity of tested repellents against the gold standard repellent DEET
- Looking for new repellents as an alternative to the current product availability

Chapter 5: CUTICULAR AND INTERNAL CHEMICAL COMPOSITION OF THE BITING MIDGES *CULICOIDES OBSOLETUS* AND *C. LUPICARIS* POTENTIAL VECTORS OF THE BLUETONGUE DISEASE

- Report the first complete description of the cuticular (external and internal) chemical profile of some species of *Culicoides*
- Determine whether different physiological stages have different chemical composition in *C. lupicaris* and *C. obsoletus* species
- Attempts to found the existence of volatile compounds emitted by *C. nubeculosus* and *Culicoides obsoletus* (Appendix IV).

RESUMEN

El género *Culicoides* (Diptera: Ceratopogonidae) engloba un extenso grupo de diminutos mosquitos de distribución mundial y ampliamente conocidos en el sector médico-veterinario por actuar como vectores de enfermedades que causan daños a animales. Excepcionalmente, en ciertas regiones tropicales de América y África están implicados como primeros agentes en la transmisión a los humanos de dos enfermedades: el virus Oropouche y la parasitosis mansonelosis.

En cuanto a la biología del género, hay que resaltar que su actividad de vuelo se desarrolla al atardecer y/o durante la noche preferiblemente, momento que es aprovechado por las hembras de *Culicoides* para picar a sus hospedadores favoritos y obtener la sangre necesaria antes de la puesta de huevos. Los lugares de ovoposición y desarrollo de los estadios inmaduros comprenden una amplia gama de sustratos húmedos o semi-acuáticos que son explicados en profundidad en la presente tesis. Aunque las especies más relevantes son aquellas con preferencias alimentarias mamofílicas, las hay ornitofílicas e incluso indiferentes u oportunistas en función del hospedador disponible en cada momento.

La importancia de los *Culicoides* radica en actuar como vectores de diferentes patógenos en animales tales como nematodos y protozoos pero más comúnmente de virus. Se conocen hasta 66 virus transmitidos por estos mosquitos, algunos de éstos son de gran relevancia internacional por los daños económicos que supone su transmisión al ganado doméstico. Las enfermedades víricas más destacables mundialmente son el virus de la Peste Equina Africana (VPEA) de los caballos, el virus de la Lengua Azul (VLA) causante de la fiebre catarral ovina de las ovejas, cabras y vacas, el virus de la Enfermedad Epizoótica Hemorrágica (VEEH) de los ciervos, el Virus Akabane (VAKV) de los rumiantes, la Fiebre Efímera Bovina (VFEB) y el Virus Palyam (VPAL). Se destaca también la reciente aparición de un nuevo virus no conocido para la ciencia que han denominado virus Schmallenberg (VSB) y que está causando grandes daños en el ovino de gran parte del continente Europeo, reportándose el primer foco en el territorio español el 13 de Marzo del 2012.

Aunque se conocen mundialmente más de 32 especies de *Culicoides* que pudieran actuar como vectores de la enfermedad de la Lengua Azul (LA), y al menos 26 serotipos diferentes (cinco de ellos en España), a nivel Europeo se incluyen las siguientes especies de *Culicoides* como los vectores más eficaces: *Culicoides imicola* ha sido catalogada como el vector más importante de la LA y de la PEA en el continente Africano así como en toda la región Mediterránea Europea. Desde la expansión de la LA a gran parte de Europa, otras especies de *Culicoides* infravaloradas como vectores han tomado gran importancia compitiendo en algunos casos con *C. imicola*, como es el caso de *C. obsoletus*, *C. scoticus*, *C. dewulfi* y *C. chiopterus* dentro del subgénero *Avaritia* y *C. lupicaris* y *C. pulicaris* dentro del subgénero *Culicoides*. El papel de otras especies potenciales como posibles vectores de la enfermedad, (e.j. *C. nubeculosus* y *C. punctatus*) no es hasta ahora lo suficientemente conocido en la comunidad científica.

De enorme importancia veterinaria, los *Culicoides* en España son vectores de la Lengua Azul, enfermedad incluida en el listado de enfermedades peligrosas de la OIE (Organización Mundial de Sanidad Animal) en vigor en el año 2013. Se trata de una enfermedad que ha despertado gran preocupación a las autoridades competentes por lo devastadora que puede llegar a ser, si no se toman las medidas oportunas. El primer brote histórico conocido de esta enfermedad tuvo lugar en 1956 en Badajoz procedente de un brote de Portugal, correspondiente al serotipo-10. La enfermad rápidamente se extendió a varias provincias cercanas del centro y sur Peninsular hasta que finalmente en el año 1960 fue erradicada gracias a extensas labores de desinfección y vacunación, con la consecuente pérdida de más de 179.000 cabezas de ganado ovino. Casi 40 años después, la enfermedad reapareció (serotipo-2) en las Islas Baleares y posteriormente en 2003, un nuevo brote esta vez provocado por el serotipo-4 afectó a Menorca. Apenas un año después, y sin previo aviso, la enfermedad alcanzó la Península Ibérica (serotipo-4), extendiéndose a gran parte del territorio español acabando con la vida de miles de cabezas de ganado y provocando grandes pérdidas económicas en el sector de la ganadería. Por otra parte, la PEA se trata de la segunda enfermedad más destacable en España, también transmitida por el género *Culicoides*, y aunque es una enfermedad endémica del centro de África, se registraron brotes en España en el año 1966 y de 1987 a 1990.

Tres años después de detectarse la LA en la Península Ibérica, ésta hace su primera aparición en el País Vasco, Navarra y Pirineos atlánticos franceses a finales del año 2007. En concreto el primer brote en el País Vasco fue detectado en Noviembre de este mismo año en el valle de Oiartzualdea (Guipúzcoa) y correspondió a un nuevo serotipo, el 1. En poco tiempo, en enero del año 2008, nuevos focos afectaron a Cantabria, Galicia y territorios limítrofes esta vez ocasionados por el serotipo-8 (procedente de ganado infectado de Alemania), ambos serotipos fueron comunes en el norte Peninsular. Desde los años 2007 al 2009 un total de 546 focos fueron notificados aunque afortunadamente desde marzo del 2009 ningún brote más ha sido reportado en el territorio vasco. Las campañas de vacunación probablemente jugaron un papel muy importante en su disminución paulatina, con el consecuente fin de los brotes epidemiológicos. Aunque en un primer momento se pensó que la LA (serotipo-1) podría haber llegado por la importación de ganado infectado con el virus de zonas afectadas, posteriores estudios basados en modelos climáticos, sustentan la hipótesis de que masas de viento procedentes de comunidades situadas al sur peninsular con poblaciones asentadas de *C. imicola*, hayan sido la ruta de llegada y actividad de vectores infectados (*C. imicola*) como forma de introducción del VLA en los meses de agosto y octubre del 2007 a Guipúzcoa. Además, el equipo de Neiker-Tecnalia registró la captura de varios ejemplares de esta especie en los focos de origen (establos) donde ocurrieron brotes de la enfermedad.

El conocimiento epidemiológico de este género de mosquitos como vectores, era escaso en todo el territorio Europeo, excepto en aquellas regiones más mediterráneas acostumbradas a sufrir brotes en diferentes periodos históricos. En lo que respecta a la ecología y biología, no se conocían estudios exhaustivos sobre la fauna de *Culicoides* y por tanto eran prácticamente desconocidos en gran parte de la geografía española con únicamente algunos trabajos publicados en el último tercio del siglo XX por diversos autores. Los primeros datos faunísticos actualizados fueron difundidos por el grupo de investigación balear, seguidos de Cataluña y País Vasco. Conjuntamente se dieron a conocer los primeros datos de composición específica de este género así como la confirmación de la presencia de hembras de *Culicoides* con el virus de la LA en granjas del País Vasco. Paralelamente a los grupos de investigación mencionados, en el año 2004 se puso en marcha el Programa Nacional de Vigilancia Serológica y Entomológica

frente a la Lengua Azul para todo el territorio de España. Este programa ha permitido conocer diversos aspectos sobre los *Culicoides* en todo el país.

Tras esta introducción sobre el grupo, a continuación se explica brevemente la metodología, resultados y conclusiones más relevantes obtenidos en la presente tesis:

CAPITULO 1 y 2 - La llegada del brote de LA al País Vasco motivó el establecimiento de un programa de seguimiento y captura de *Culicoides* por parte del equipo de Neiker-Tecnalia. El programa se llevó a cabo mediante la colocación de trampas a finales del año 2007 en las granjas afectadas por el primer foco de LA en el valle de Oiartzualdea y durante los años posteriores éste fue implementándose en otras granjas situadas en diferentes localidades de la Comunidad Autónoma del País Vasco.

Los ejemplares de *Culicoides* se muestrearon mediante el empleo de trampas de aspiración con luz ultravioleta (CDC light trap, modelo 1212). Los mosquitos son atraídos por la fuente de luz, aspirados por un ventilador y conducidos a través de un sistema de embudo de tul a un extremo inferior dotado de un bote recolector relleno parcialmente de un líquido conservante que almacenan los ejemplares hasta su recogida. Para el programa de seguimiento se llevó a cabo un estudio anual en un total de 11 granjas con ganado ovino situadas en diferentes áreas bio-geográficas, con un mínimo de 80 cabezas de ganado y que preferiblemente hubieran estado afectadas por brotes de la LA. Se usaron dos trampas simultáneamente por granja, una colocada en el exterior (pastizal) y otra en el interior del establo. La región de estudió se dividió por provincias: Guipúzcoa (tres granjas muestreadas en el año 2008), Álava (cinco granjas en el año 2009) y Vizcaya (tres granjas en el año 2010). Dichas trampas se hicieron funcionar nocturnamente de forma continua durante todo el año correspondiente y sirvieron para conocer la biodiversidad. Adicionalmente en el año 2011, se seleccionaron siete ecosistemas naturales con fauna salvaje para ser también muestreados, esta vez con trampas CDC portátiles y durante un periodo de 6 meses, 2 días de captura por semana.

El programa entomológico de *Culicoides* en las tres provincias vascas permitió dar a conocer interesantes y diversos aspectos sobre su biología, diversidad, fenología, dinámicas poblacionales, distribución y composición específica. Hasta un total de

348.685 ejemplares pertenecientes a 52 especies fueron capturadas en todo el muestreo, 47 de ellas en las granjas de ovino y 31 en los ecosistemas naturales. En general, el género *Culicoides* fue abundante en hábitats asociados con las explotaciones ganaderas ovinas, en los que en óptimos días calurosos de verano llegaron a registrarse capturas de hasta 5.000-10.000 ejemplares por trampa/noche. El número total anual de individuos capturados varió considerablemente según el tipo de granjas, con capturas que oscilaron desde los 1.500 ejemplares en granjas con poca abundancia hasta los 140.0000 en aquellas con mayor abundancia. Entre el 70-90% de las capturas de *Culicoides* se correspondieron con dos especies morfológicamente similares agrupadas en un complejo de especies llamado Obsoletus que incluye a *Culicoides obsoletus* y *Culicoides scoticus.* La similitud morfológica de las hembras de ambas especies implicó que sólo fueran diferenciables fehacientemente atendiendo a ciertos caracteres de los machos, aunque recientes estudios permitieron separar las hembras de ambas especies con cierta precisión sin recurrir a técnicas moleculares. Atendiendo a los machos, así como a sub-muestras de hembras, se determinó que ambas especies aparecen en todas las granjas, aunque *C. obsoletus* fue mucho más abundante. El segundo grupo de especies más numeroso se trataba del complejo Pulicaris (*C. lupicaris* y *C. pulicaris*) que según provincias osciló entre un 3,5% a un 14% del total de capturas.

Ambos complejos de especies mencionados fueron habituales en todas las granjas estudiadas aunque su distribución temporal varío según localidades geográficas. *Culicoides obsoletus/scoticus* y *C. lupicaris/pulicaris* fueron capturados durante todo el año e incluso durante el invierno en las provincias de Guipúzcoa y Vizcaya (regiones de influencias atlánticas con inviernos suaves) mientras que en algunas granjas de Álava (región con inviernos fríos y frecuentes heladas) ningún *Culicoides* fue capturado durante los tres meses invernales más duros (diciembre, enero y febrero). Con carácter general, la mayoría de especies fueron activas de mayo a noviembre, con picos máximos poblacionales entorno a los meses de verano. En algunas granjas se observaron dos picos poblacionales claros, uno largo que transcurre durante los meses de abril-agosto y otro en septiembre-noviembre de menor amplitud y abundancia, mientras que a veces este último fue imperceptible. La presencia de *C. imicola* se restringió a unos pocos ejemplares capturados en Guipúzcoa, un solo ejemplar en Vizcaya y ninguno en Álava. La distribución de *C. nubeculosus* se limitó a las provincias norteñas con climas suaves, especialmente en Vizcaya. Respecto al comportamiento de los *Culicoides* (trampas

exteriores vs trampas interiores), se observó que un 65,9% de las capturas ocurrieron en trampas externas y la abundancia de especies fue entre 2-2,5 veces más rica en trampas externas que en internas, siendo estas diferencias menos acusadas en Vizcaya.

Los resultados obtenidos del estudio de los siete ecosistemas naturales es de gran interés ya que la mayoría de los muestreos entomológicos suelen centrarse en granjas de explotación ganaderas. Las especies capturadas más abundantes fueron marcadamente diferentes de las obtenidas en las granjas, con *C. festivipennis*, *C. alazanicus* y *C. brunnicans* englobando el 48,5% del total de capturas. El ecosistema más rico fue la charca. La distribución de estas especies varió según el ecosistema, pero se observó que las dos primeras especies podrían estar asociadas a hábitats con presencia de abundantes aves, de las cuales muy probablemente se alimentan. Curiosamente en los hábitats naturales la presencia de *C. obsoletus/scoticus* es notablemente menor, con capturas que variaron entre el 0,5 al 10% del total de capturas. La proporción de *C. scoticus* respecto a *C. obsoletus* basada en la identificación de los machos, fue un poco mayor que en las granjas, en torno al 25%. En relación a las dinámicas poblacionales, mayo fue el mes con más capturas con un descenso durante los meses siguientes hasta que en septiembre se observó un aumento en las capturas, muy probablemente atribuible a las lluvias acontecidas en este mes.

Es importante recalcar que el entendimiento de la bio-ecología de estos mosquitos es muy complejo ya que existen multitud de factores que se interrelacionan entre sí en un ambiente común, y por ello algunos aspectos deben ser tenidos en cuenta en la interpretación y comparación de los resultados como son: características físicas de los establos, número de cabezas de ganado, prácticas y manejo del ganado, almacenamiento y procesado de los residuos animales, pastoreo, presencia de lugares de cría apropiados, visibilidad de las trampas, factores climatológicos: temperatura, humedad relativa, régimen y periodicidad de lluvias, viento, estacionalidad etc.

CAPITULO 3 - El estudio de los lugares de desarrollo de los estadios inmaduros de los *Culicoides* es de gran interés por varios motivos. El principal es que su conocimiento en profundidad podría tener importantes implicaciones como enfoque práctico para el control de estos vectores centrando su lucha (biológica y/o química) en los estadios inmaduros en lugar de en los estadios adultos. Hasta el momento, el conocimiento de los

hábitats de las larvas de muchas especies de *Culicoides* ha sido bastante desconocido. Curiosamente los hábitats de especies generalmente comunes, como son aquellas pertenecientes al grupo Obsoletus y Pulicaris, no habían sido lo suficientemente caracterizadas en profundidad, reportándose trabajos de investigación infructuosos en la búsqueda de éstos.

Para llevar a cabo un estudio riguroso de los lugares de cría se eligió la granja de Elguea (Álava), entre otros motivos, por haber mostrado en estudios preliminares alta diversidad y haber sufrido los efectos de la LA. Un total de 17 micro-hábitats posibles de albergar estadios inmaduros de estos mosquitos fueron elegidos en el entorno de la granja y sus alrededores. Se recogieron quincenalmente muestras de estos sustratos durante todo un año y se introdujeron en dispositivos a la espera de su emergencia y tras 30-35 días de su muestreo, los botes recolectores fueron vaciados para la identificación de los ejemplares. Al mismo tiempo, una trampa de luz fue colocada en el centro del pastizal de la granja y se recogió su contenido en los mismos días que en los sustratos de suelo.

Se identificaron un total 37 especies (66.569 individuos) en trampas de luz y 28 especies (11.396 ejemplares) emergidos de los sustratos de micro-hábitats. *Culicoides obsoletus* y *C. scoticus* (agrupados juntos) fueron particularmente abundantes tanto en las trampas de luz como en los sustratos, 58,6 y 74,5% del total de capturas respectivamente. Ambas especies, fueron encontradas criando preferiblemente en dos hábitats. En el caso de *C. obsoletus sensu stricto,* el estiércol maduro (entre 1 y 2 años de fermentación), así como estiércol con materia orgánica depositado en los bordes del establo y el estiércol fresco (días/semanas de fermentación), todos ellos depositados al aire libre, fueron los hábitats donde emergieron la gran mayoría de los ejemplares capturados. Se han registrado muestras con estiércol maduro (314^3 cm de volumen) de los cuales emergieron más de 1.200 especímenes de *C. obsoletus.* En segundo lugar, los hábitats de cría de *C. scoticus* s.s. y *C. lupicaris*, estaban asociados con la hojarasca en descomposición situada en lugares frescos y sombríos de los alrededores de la granja, donde se desarrollaba la planta *Lathraea clandestina.*

Otras especies como *C. festivipennis, C. punctatus* y *C. brunnicans* fueron especialmente abundantes en los márgenes de la charca situada al fondo del pastizal.

Entre los ecosistemas más ricos en especies se destacan los márgenes húmedos asociados a la charca así como a la zanja herbácea saturada de agua que se forma por el goteo del agua del tejado en el borde del pastizal. Los ecosistemas del estiércol y de la hojarasca a pesar de albergar más del 70% de todas las capturas son escasos en número de especies.

Por el contrario, en cinco ecosistemas (musgo forestal, excrementos de oveja, cama del interior del establo, estiércol disperso en el pastizal y acúmulo arcilloso del río) no se obtuvieron ejemplares de *Culicoides*. Muestreos adicionales llevados a cabo a diferentes profundidades y gradientes de humedad, mostraron que los *Culicoides* se distribuían junto al borde del agua (49,4%) y a medida que se recogían muestras en sustratos más secos, a 50 cm (32,4%) y a un metro del borde del agua, se recogieron aún menos ejemplares (18,2%). El estudio en relación con la profundidad indicó que los ejemplares de *Culicoides* se ubicaban en las capas más externas (0-3 cm) excepto en los ecosistemas de estiércol que lo hacían en capas más profundas (> 6 cm). Los picos de abundancia ocurrieron tanto en la trampas de luz como en los sustratos en los meses de verano, con cierta correspondencia entre ambos, excepto en los meses de otoño, donde sólo se capturaron ejemplares con las trampas de luz y no en los micro-hábitats.

A raíz de los resultados obtenidos, *C. obsoletus* parece presentar una fuerte asociación con los hábitats ligados al ganado, siendo particularmente común en montículos de estiércol maduro almacenados en el exterior de las instalaciones ganaderas. Se trata de una especie versátil ya que tiene la capacidad de criar en gran variedad de sustratos, aunque el número de ejemplares capturados en otros hábitats no estercoleros, es notablemente bajo. Por otra parte su especie afín, *C. scoticus* parece ocupar una distribución más limitada, prefiriendo ocupar hábitats forestales con hojarasca en descomposición. Las especies del grupo Pulicaris fueron capturadas en una gran variedad de ecosistemas, particularmente criando en zonas húmedas de la charca y en la zanja de goteo, aunque *C. lupicaris* es bastante más cosmopolita ocupando otros micro-hábitats diferentes. El papel de *C. punctatus*, una especie común en los hábitats anteriormente señalados, ha sido generalmente subestimado en su papel como vector, pero recientemente se ha sugerido que podría actuar como transmisor de nuevas enfermedades, tales como el virus VSB. La toma de una serie de medidas simples y concretas por parte de los propietarios ganaderos podría evitar en cierta medida aquellos

hábitats más propensos para la cría de estos mosquitos impidiendo o dificultando su cría masiva. Así, se deberían de evitar el acúmulo de materia orgánica en las entradas a los establos y charcos permanentes con materia orgánica. El uso de compostadoras, acidificación del estiércol y/o lonas cubriendo el estiércol, y/o instalación o mejora en las cañerías por donde circula el agua procedente del tejado y derivarlas a depósitos evitando el goteo, son algunas medidas posibles a implementar, entre otras.

CAPITULO 4 - La aparición de imprevisibles y desconocidas enfermedades como ha sido el caso muy reciente del virus Schmallenberg, da lugar a replantearse nuevas estrategias de control alternativas al uso de vacunas, las cuales requieren de un cierto tiempo para su fabricación y puesta a punto antes de ser utilizadas. En este sentido, el desarrollo de potentes repelentes con un prolongado efecto de protección puede servir como alternativa provisional para disminuir las probabilidades de picadura y por tanto de transmisión. Es importante recordar que el rol de los *Culicoides* no solo radica en actuar como vectores de enfermedades sino también por la propia molestia que sus picaduras causan tanto a animales como a humanos, especialmente en algunas regiones del mundo donde son tan abundantes que terminan perjudicando al turismo y al desempeño de labores al aire libre. Por otra parte, el uso del DEET, uno de los más conocidos y usados repelentes en el mundo, está planteando cada vez más problemas en el ámbito de la salud pública. La búsqueda de alternativas y de nuevos repelentes se hace necesaria y en este campo ha adquirido gran interés los repelentes derivados de extractos de plantas, por ser en general más seguros y menos tóxicos que los sintéticos. Aunque la evaluación de repelentes directamente sobre animales sería el método más realista, éste puede conllevar problemas (desconocida toxicidad, reacciones en la piel, dificultades de evaluación, manejo del ganado…), su estudio mediante pruebas de laboratorio o sobre trampas u otros objetos es también válida como primer método de evaluación.

La forma de evaluar los repelentes se basó preliminarmente en ensayos de laboratorio: olfactómetro de dos vías y pruebas de posado en filtros impregnados de repelente dentro de cajas de mosquitos. En segundo lugar, los repelentes que resultaron ser más alentadores fueron valorados en pruebas de campo. La metodología usada en campo se basó en aquella seguida por otros autores, aunque con algunas modificaciones puntuales. En resumen, una porción de malla anti-mosquitos de ± 2 mm de luz de malla y 0,15 m^2, la cual permitió el paso libre de los *Culicoides* por su diminuto tamaño, fue

impregnada con la solución de repelente a evaluar y se fijó alrededor de las trampas de luz tipo CDC con luz blanca. Los repelentes fueron testados en cuatro tandas independientes cada uno con un control (alcohol puro) y el repelente DEET como repelente estándar durante las 5 primeras horas de la noche.

Los resultados obtenidos del estudio de 23 compuestos (19 repelentes y cuatro mezclas de ellos), tanto en ensayos de laboratorio como de seis de estos en pruebas de campo resultaron ser muy prometedores para una mezcla de ácidos grasos orgánicos (C8, C9, C10) en la proporción 1:1:1, los cuales en muchos casos resultaron repeler incluso a un número mayor de *Culicoides* que el DEET. En concreto, las trampas con ácidos orgánicos a las dosis 10% y 25% capturaron 2,2 veces y 3,6 veces menos de ejemplares respectivamente que las trampas control. En el caso del DEET entre 2-2,2 y 2,7-3,2 veces de reducción al 10% y 25% respectivamente. La mezcla de geranil acetona más 6-methyl-5-hepten-2-ona (1:1) no resultó ser eficaz a ninguna de las concentraciones evaluadas, al contener un número de capturas similar a la trampa control. De las dos dosis testadas, la mayor dosis (25% de concentración de repelente) funcionó notablemente mejor que la del 10%. Aunque los repelentes naturales (lavanda, eucalipto, jazmín y la mezcla 1:1:1 de romero, jazmín y lavanda) redujeron a la mitad el número de capturas frente al control, solo el eucalipto de limón a la dosis del 25% redujo significativamente el número de *Culicoides* spp. (2,7 veces menos). A pesar de que la mayoría de las especies de *Culicoides* capturadas respondieron de forma similar a los repelentes, el subgénero *Silvaticulicoides* parecía ser más resistente a la acción de los repelentes. Por todo ello, se destaca la prometedora acción repelente frente a mosquitos del género *Culicoides* de los ácidos grasos, del DEET y del eucalipto de limón.

La investigación de nuevas mezclas con efectos sinérgicos así como de nuevos dispositivos de emisión más prolongada son objetivos que deberían alcanzarse a corto plazo en este ámbito.

CAPITULO 5 - El estudio de la cutícula de los insectos tiene gran interés desde varios puntos de vista. En primer lugar, se trata de una herramienta adicional utilizada con fines taxonómicos e identificación. Está bien documentado el hecho de que complejos de especies morfológicamente similares pueden tener un perfil químico cuticular diferente, y éste puede ser utilizado para distinguir especies de una población e incluso está siendo

utilizado para datar la edad de especies de mosquitos (Culícidos) en función de la cuantificación de ciertos compuestos químicos. Esto es de gran valor para estudios epidemiológicos en los que conocer la edad de un mosquito vector es esencial para calcular la probabilidad de transmisión de un patógeno. En segundo lugar, el conocimiento de los compuestos que integran la parte más externa de la cutícula pueden servir para conocer mejor los mecanismos de adhesión e invasión de patógenos tales como bacterias y hongos entomopatópogenos. Esto es, según los compuestos epi-cuticulares presentes (ej. ciertos ácidos grasos), la invasión de patógenos será viable o por el contrario impedida. En tercer lugar, muchos de los procesos biológicos y fisiológicos de la comunicación química tanto intra-especifica como inter-específica están mediados por esta capa cuticular: procesos de comportamiento, cópula, producción de feromonas, hormonas de alarma, etc.

Así como el conocimiento de la composición cuticular de algunas especies de mosquitos culícidos ha sido estudiada en frecuentes trabajos, la familia Ceratopogonidae y en concreto el género *Culicoides* ha sido parcialmente olvidado, con únicamente dos trabajos antiguos publicados que hacen referencia a los ácidos grasos y los hidrocarburos de varias especies Asiáticas y Australianas respectivamente. Se eligieron algunas de las especies más comunes de *Culicoides* y una especie de *Forcipomyia* (subfamilia Forcipomyiinae) para ser analizados sus perfiles químicos tanto de la capa externa de su cutícula como de la parte interna mediante la técnica de Cromatografía de Gases acoplada a Espectrometría de Masas (GC/MS). Las dos especies de mayor interés médico-veterinario (*C. obsoletus* y *C. lupicaris*) fueron estudiadas en mayor profundidad.

Se destacaron un total de 69 compuestos (57 externos y 43 internos) pertenecientes a siete clases químicas, entre los más comunes se encontraron los siguientes: ácidos grasos (C6-C20), con C16:0, C16:1 y C18:1 especialmente abundantes, hidrocarburos lineales (C15-C33) siendo los compuestos pares más abundantes, hidrocarburos ramificados (C29-C38), terpenos (escualeno), esteroides (colesterol), aldehídos (C20-C30), ésteres y otros compuestos como constituyentes menores. En función de la capa estudiada, mientras que el contenido relativo de los hidrocarburos lineales, ramificados y aldehídos era alto en los extractos cuticulares, éstos eran minoritarios o ausentes en tejidos internos. Por el contrario, el contenido de

los ácidos grasos, terpenos y esteroides era en general mayor en las capas internas. El análisis cuantitativo de los cuatro estadios gonotróficos estudiados de *C. obsoletus* y *C. lupicaris* (nulíparas, páridos, con sangre y grávidos) no resultaron ser significativamente diferentes entre ellos, excepto en el contenido de escualeno y colesterol.

El papel que podrían desempeñar algunos de los compuestos detectados en otras especies de *Culicoides* así como en otras especies similares de mosquitos u otros insectos, es discutido en el presente capítulo.

ANTECEDENTES AL INICIO DE LA TESIS

La inesperada llegada de la enfermedad de la Lengua Azul a la Comunidad Autónoma del País Vasco en Noviembre del año 2007 desencadenó una serie de objetivos y acciones a llevar a cabo por parte del equipo entomológico de Neiker-Tecnalia.

1.- Optimización de las trampas de *Culicoides* (Ceratopogonidae) para que éstas capturen el máximo número de insectos hematófagos en los establos.

2.- Comprobar la eficacia de trampas italianas específicas de dípteros Culicidae frente a las trampas CDC-UVA de *Culicoides*.

3.- Ensayo de posibles sustancias repelentes de origen natural frente a los jejenes para su posible aplicabilidad en el animal.

4.- Estudio de las dinámicas poblacionales de las especies de *Culicoides* ligadas a los establos de Guipúzkoa en los que apareció el primer foco de lengua azul en 2007.

5.- Aislamiento del virus de la LA del interior de los diferentes estadios gonotróficos de varias especies de *Culicoides* (complejo Obsoletus y Pulicaris).

6.- Detección de la presencia de ejemplares de *Culicoides imicola* en las granjas donde se detectaron brotes de LA.

Fruto de algunas de estas acciones se dieron a conocer a la comunidad científica los primeros datos acerca de diversos aspectos de los *Culicoides* en el País Vasco, a través de dos publicaciones científicas (Goldarazena et al. 2008) y (Romón et al. 2012).

En la primera publicación mencionada se informó por primera vez acerca de la composición específica de los *Culicoides* en aquellas granjas afectadas por los primeros brotes de LA, reportándose la presencia de varios ejemplares de *Culicoides imicola.* Tales especímenes fueron capturados mediante trampas de luz entre los meses de Noviembre y Diciembre de 2007 en las granjas de Zabaltxo y Oiantzabal (Guipúzcoa).

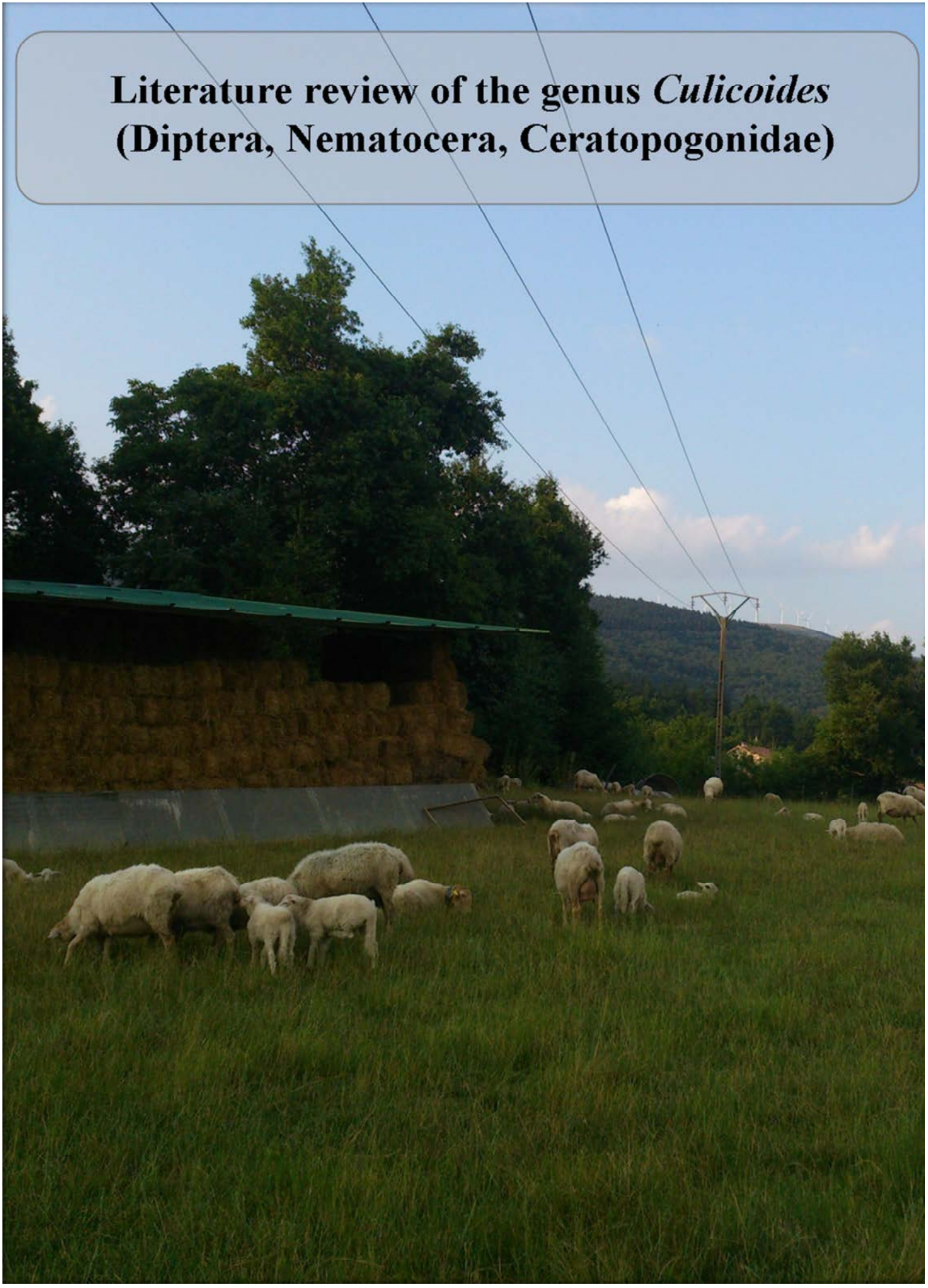
La segunda publicación, se centra en estudiar las dinámicas poblacionales, fenología y composición específica de diversas especies de *Culicoides* en granjas guipuzcoanas. Asímismo, se estudiaron una serie de atrayentes (acetona y ácido láctico)

incorporados a trampas de luz con el objetivo de optimizar las capturas de *Culicoides,* y así reducir sus poblaciones. Y finalmente, aislaron mediante la técnica RT-PCR (reacción en cadena de la polimerasa con transcriptasa inversa) el virus de la LA de "pools" de *Culicoides.* A partir de este trabajo, se confirmó la presencia del virus tanto en el complejo Obsoletus como en el grupo Pulicaris, tanto en ejemplares páridos como en ejemplares recién alimentados de sangre. Los resultados alentadores obtenidos con los atrayentes testados en conjunción con los presentados en esta tesis con repelentes (**Chapter 4**) pueden servir para implentar estrategias de tipo "push-pull" (veáse sección 12 de la introducción).

Bajo este contexto nace la tesis de
Mikel A. González González de Heredia

INTRODUCTION

Literature review of the genus *Culicoides* (Diptera, Nematocera, Ceratopogonidae)



LITERATURE REVIEW OF THE GENUS *CULICOIDES* (DIPTERA, NEMATOCERA,CERATOPOGONIDAE)

1. TAXONOMIC HIERARCHY

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*

Although the systematics and taxonomy of this genus are confused, the subgeneric classification of *Culicoides* species was reported by Borkent (2012a), in whose list he reported that the genus *Culicoides* is placed in 31 subgenera and 38 species groups (unplaced to subgenus). Borkent (2012a) also mentioned that at least 13% of the *Culicoides* world fauna are not placed in any group and usually are included as miscellaneous species, most likely *nomina dubia.*

In contrast, European species are more restricted with a total of nine defined subgenera and a group of unplaced species, although the afrotropical subgenus *Synhelea* is not included by some authors (Meiswinkel et al. 2004; Szadziewski and Borkent, 2004):

- Subgenera *Culicoides* Latreille, 1809; *Oecacta* Poey, 1853; *Pontoculicoides* Remm, 1968; *Silvaticulicoides* Glukhova, 1972; *Wirthomyia* Vargas, 1973; *Avaritia* Fox, 1955; *Beltranmyia* Vargas, 1953; *Monoculicoides* Khalaf, 1954; *Synhelea* Kieffer, 1925 and unplaced subgenus (this may include various subgenus of confused classification).

2. HISTORICAL REVIEW

2.1. Adult stages

The first account of adult *Culicoides* stages was given as early as 1713 by the Reverend W. Derham, who described a small gnat which he named *Culex minimus nigricans maculatus sanguisuga* (Edwards, 1939). Some years later, in 1758, Carl Von Linné (Linnaeus) described for first time the species *Culex pulicaris* in his seminal work, *Systema naturae*. Now, this species is known as *Culicoides pulicaris.*

In 1800, the name *Helea* was published for the first time to refer to these midges, without any species being mentioned, and this term was quickly rejected by nearly all dipterists in this field (Edwards, 1920). Meigen in 1803, named the genus *Ceratopogon* and most entomologists preferred to use this term, until in Paris in 1809, Pierre André Latreille established the genus *Culicoides* and defined it in his book "*Genera crustaceorum et insectorum*". Although the descriptions were perfect, during the following years, most 19th century dipterists preferred the name *Ceratopogon*, instead of *Culicoides.* For this reason, several species of *Culicoides* (Ceratopogonidae: family) were erroneously described initially as *Ceratopogon* and/or *Helea* (Heleidae: family) causing several problems and confusions in the later years. It was at the beginning of the 20,th when Kieffer (1901) broke up with the old genus *Ceratopogon* and the genus *Culicoides* created by Latreille reappeared and was finally included within the family Ceratopogonidae. Hence, the name Ceratopogonidae derived from *Ceratopogon* and not from *Culicoides*. At that time, these insects seemed to be considered no more than a nuisance, which probably explains why Blanchard (1890) did not include them in his "*Traité de zoologie médicale*". In contrast, veterinarians were more interested and included various biting midges species in the "*Synopsis de Parasitologie de l'home et des animaux domestiques*" by Gedoelst (1809) (review of Houin, 2008).

Relevant contributions throughout the first part of 20th century by Malloch (1917) and Kieffer (1925) enabled the organization of some generic names and the establishment of the superfamily Chironomoidea, composed of two families: Chironomidae and Ceratopogonidae. The great number of *Culicoides* species described in short time from all around the world, required the collection of the species within the

genus into morphologically defined clusters, a task which was achieved by Macfie (1940), (rewiew of Houin, 2008). The most important authors that have contributed to the knowledge about the biting midges from the different geographical regions in the world are listed below.

- Past century:

i) Palaearctic region: M. Goetghebuer, (Belgian species), J.J. Kieffer, F.W. Edwards (French and British species). In mid-twenties, D.S. Kettle, J.A. Downes, J.W.E. Lawson, J.A. Campbell and E.C. Pelham-Clinton (Britain species), J. Callot and M. Kremer (French area). More recently, J.C. Delécolle contributions in taxonomy have allowed the identification of new several species of *Culicoides* from France.

ii) Russian region: S.M. Dzhafarov, V.M. Glukhova, A.V. Gutsevich and H. Remm have extended significantly knowledge of those areas.

iii) Neotropical region: valuable taxonomical contributions have been made by F.A.S. Barbosa, I. Fox, J. Lane, A. Lutz, J.W.S. MacFie, I. Ortiz, L. Vargas, W.W. Wirth, R.H. Jones and F.S. Blanton.

iv) African region: H.F. Carter, O.G. Fiedler and J. Clastrier (African Ceratopogonidae) and A. Ingram, J.W.S. MacFie, B. de Meillon and O.G.H. Fiedler (Ethiopian Region).

v) Australian region: this area was principally monographed by Lee and Reye (1953).

vi) Oriental region: Asian countries have been ignored by ceratopogonid workers and, with the exception of a reference by Causey (1938) to *Culicoides* species occurring in Siam, little is known of the *Culicoides* of this region. M. Tokunaga and P. Arnaud (Japan and surrounding areas in the mid-twenties).

-Nowadays:

Art Borkent, a world expert on various groups of dipterans, has published a complete catalog compiling all the worldwide Ceratopogonidae species (extant and fossil). It is available and periodically updated in: http://wwx.inhs.illinois.edu/research/flytree/borkent/). Another useful source of information can be consulted in: http://www.faunaeur.org/full_results.php?id=11644. Here, the distribution and taxonomy of the European *Culicoides* species have been summarized by Szadziewski and Borkent (2004). It is worth noting the considerable involvement of Jean Claude Delécolle in identifying many species, mainly from European countries. Similarly, the teamwork of Gustavo Spinelli, an expert taxonomist in the Neotropical region, is carrying out the identification of several unknown ceratopogonid species in those areas.

In the case of the Iberian Peninsula, the most important contributors to the taxonomy of *Culicoides* were S. Herráiz, G. Collado and P.S. Mellor (in the years 1982-1985), J.C. Delécolle (around 2002), V. Sarto i Monteys, S. Talavera and M. González (from 2003 onwards).

2.2. Immature stages

With reference to the early stages of *Culicoides*, it was Hill in 1947 who quoted a description of a larva and pupa of what is apparently the *Culicoides* species given by Derham as along ago as 1712. In 1913, Lutz reported some species of *Culicoides* living in specific conditions. At the same time, Goetghebuer (1919) described the larvae of *C. pulicaris.*

In 1920, Carier et al. produced their study of the Gold Coast Ceratopogonidae midges. The studies of the early stages of European species were provided by Thienemann (1928), Mayer (1934), in a compilation by Lenz (1939) and by Hill (1947), who also made an exhaustive survey of the literature and drew attention to the scarcity of larval descriptions. A complete work about early stages was provided by Kettle and Lawson (1953). Several works were published during the last part of the 21 th century, especially focusing on specific groups of *Culicoides* larvae. Currently, larvae and/or pupae are described in only 19% of the total *Culcoides* species (Borkent, 2012a).

3. TECHNIQUES FOR *CULICOIDES* DETERMINATION

i) Based on morphological characters: the most widespread method for *Culicoides* identification is by means of optical devices. Firstly, preliminary identification is usually performed with stereoscopic microscope. Secondly, the accurate identification for confusing species demands a higher magnification reached with a compound microscope. Identification is achieved following the appropriate taxonomical keys.

- **Stereoscopic microscope:** species identification based on morphological features is difficult for *Culicoides* midges. However, some important identification characters are useful. Wing pattern is especially important, as many species bear characteristic pale-dark spots along the entire wing. In contrast, some species, to a lesser extent exhibit a wing without a distinctive spot pattern. The size and shape of the maxillary palps in females are also used as a diagnostic character, despite the limited zoom-magnification of these optical devices generally not allowing to focus on specific details. In males, the genitalia is an important taxonomical character, but stereomicroscope only enables a general vision, although with practice a correct identification in some species groups is possible. The tiny imperceptible remaining morphological *Culicoides* features must be observed with microscopes.

- **Compound microscope:** suitable preparations in glass-slides are required for *Culicoides* species-level identification. Some useful characters in females are eyes (hairless, separation, location of sensilla chaetic), palpal sensory pits (shape, number and depth of sensory pits), antennal segments (distribution of sensilla coeloconic), presence of pharyngeal ornamentation, legs (spines), wing (macrotrichia), spermathecae (number and shape). In male genitalia these characters are ninth tergite apicolateral processes (size and shape), ninth tergite lobes and notch (presence/absence), ninth sternite caudomedian excavation (shape) and its membrane (bare or spiculate), aedeagus (body size and shape), parameres (shape) and apodemes of basistyle (shape and size). Some other specific features are characteristic of certain *Culicoides* species.

Moreover, the existence of cryptic/sibling species and species complexes require combined molecular and morphological approaches.

ii) Based on molecular tools: several molecular tools have been developed to identify the *Culicoides* species or to study their phylogenetic relationships (Pili et al. 2010; Deblauwe et al. 2012).

- Molecular identification is performed by two main PCR (polymerase chain reaction) techniques:

  - Real-time PCR
  - Multiplex PCR

- Many molecular tools and markers are used:

  - Internal transcribed spacer 1 (ITS1) rDNA
  - Internal transcribed spacer 2 (ITS2) rDNA
  - Mitochondrial cytocrome oxidase subunit I (COI) DNA (phylogenetic studies)
  - Isozyme electrophoresis (genetic profile)
  - Microarray technology

iii) Based on near-infrared spectroscopy (NIRS): this is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum (from about 350 nm to 2500 nm). It has been implemented in different entomological studies to determine sex, differentiate species, and detect insect pests, age-grade stored-product insects and others.

Recently it has been tested with tiny insects such as in the age-grading of *C. sonorensis* (Reeves et al. 2010). The technology is non-destructive, automated and can be tested by PCR or virus isolation.

iv) Based on MALDI-TOF mass spectrometry: this is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. The MALDI is a two step process:

First, desorption is triggered by UV and the second step is ionization (more accurately protonation or deprotonation).

This is a new technique developed with *Culicoides* species for first time. It provides a new method for *Culicoides* identification and also age-grading. It is rapid, simple, reliable and cost-effective (Kaufmann et al. 2011).

v) Based on gas chromatography-mass spectrometry (GC-MS): This is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include pheromones detection, identification of unknown compounds, profile characterization…

This technique is described in **Chapter 5** to characterize the cuticular composition of various *Culicoides* species.

4. MORPHOLOGICAL DESCRIPTION

4.1. Adults

Culicoides nematocerous flies are a group of small biting midges belonging to the Ceratopogonidae family, closely related to the Chironomidae family. *Culicoides* midges are also called jejenes, manta blanca and polvorines (in Spanish) and punkie flies, no-see-ums, biting gnats, midgies, moose flies and five-o´s in English speaking countries. *Culicoides* are also often named sandflies, but this term is more appropiate for *Phlebotomus* spp. (Diptera: Psychodidae).

They are small to medium size midges, length 1 to 5 mm and weight near to 0,5 μg, although species from Spain are usually smaller, except members of the subgenus *Monoculicoides* (Figs. 1-2). *Culicoides* midges have a characteristic arched thorax giving a “hump-backed” appearance. The wings are folded scissor-like over the body. The black and white patterning typical of the single pair of wings of most species is the first character and easiest clue for their identification (see **Chapter 1: wing pattern**). Sexes are easily recognized due to their antennae, plumose in the male and non-plumose in the female.

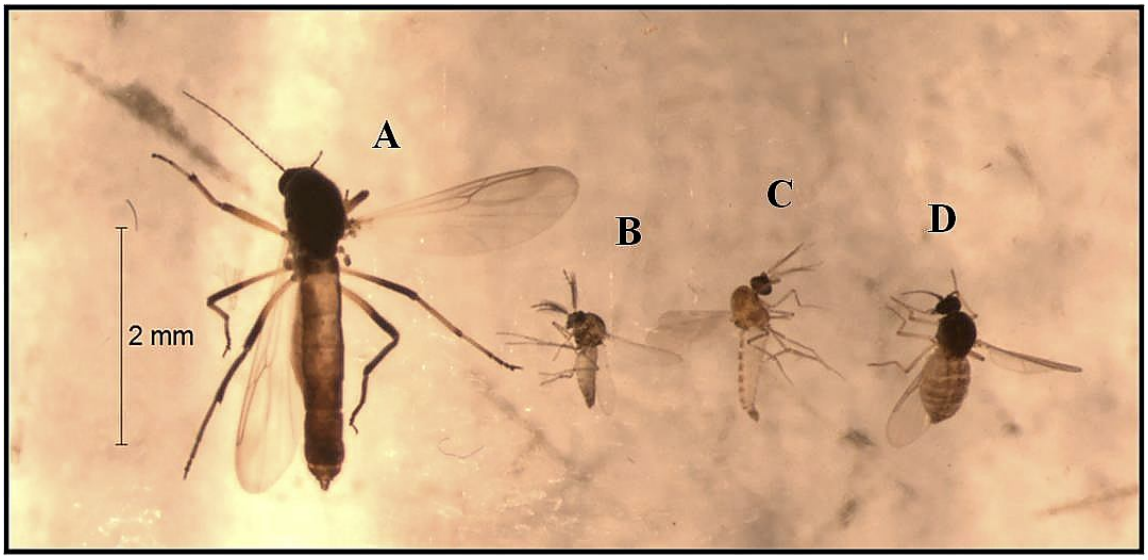


Fig. 1. Size comparison of some members of the family Ceratopogonidae **(A)** *Bezzia* spp. **(B)** *Dasyhelea* spp. **(C)** *Culicoides* spp. **(D)** *Atrichopogon* spp.

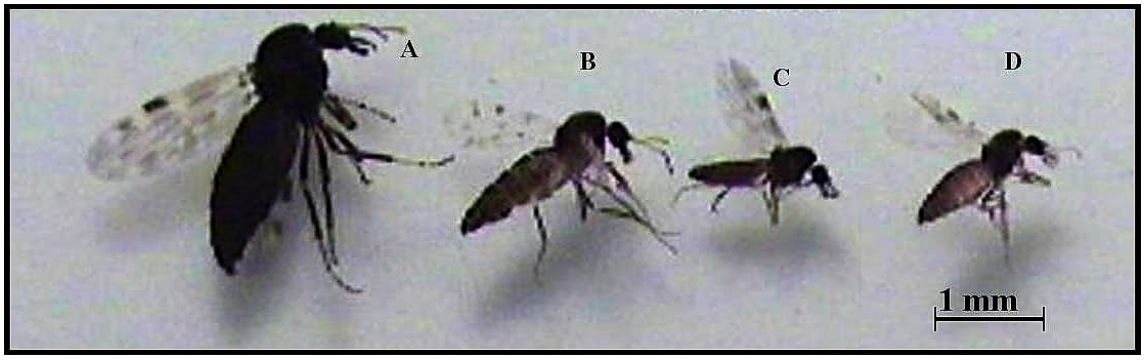


Fig. 2. Examples of different *Culicoides* species **(A)** *C. nubeculosus* **(B)** *C. pulicaris* **(C)** *C. obsoletus* **(D)** *C. dewulfi.*

Specific features of adult Culicoides: The body is moderately setaceous with weak setae and absent spines. *Mesonotum* with well-developed humeral pits, placed anteriorly on the dorsal surface of the thorax. Thorax with *scutum* spots, which sometimes are useful for species identification. Antennae bearing 15 segments and a characteristic distribution of sensilla, with sensilla coeloconica especially relevant for species identification level. Palpus with five segments, third segment variably in shape and size bearing a characteristic pattern of sensory pits. Female mouth parts modified to blood-feeding, with mandible and maxillae in the majority of the species with numerous fine teeth, in contrast to males mouth parts (Fig. 3). Slender legs, without remarkable spines; fore legs with an apical tibia with a conspicuous spine surrounded by a row of setae, medium legs without additional structures, and hind apical tibia with a comb of conspicuous spines. Fourth tarsomeres cylindrical, female and short, identical and simple male claws. *Empodium* present but small, sometimes vestigial. Wings cover the whole abdomen when resting; they usually bear a distinct pattern of pale areas on a dark background. Wings are larger and narrower in males than in females. Female abdomen is stout and bears between one to three spermathecae (two being the most common). Male genitalia are slender and more complex, bearing a "forceps-shaped" structure (basistyle and ditistyle) with different sclerotized pieces including *aedeagus*, parameres and apicolateral processes, among others [description adapted from de Meillon and Wirth (1991)]. More details are provided in the following pages.

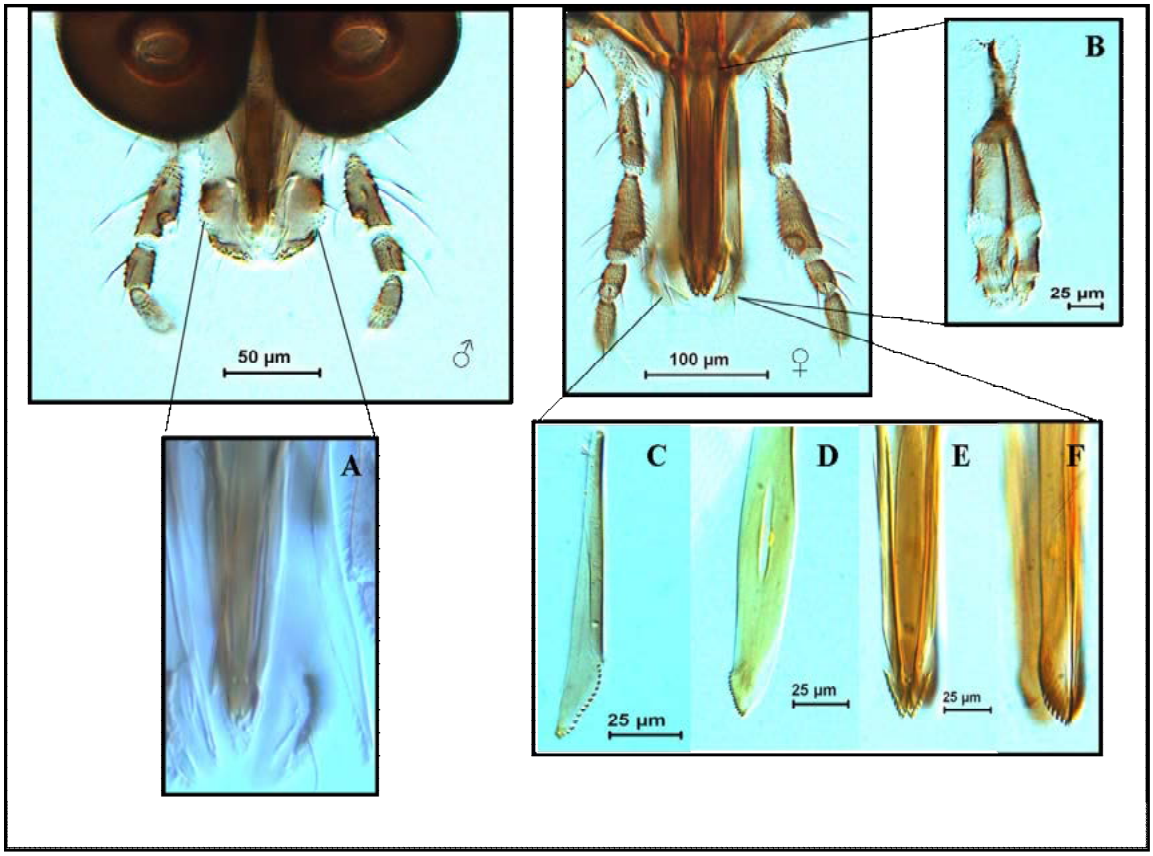


Fig. 3. Mouth parts of *Culicoides* sp. male (on the left) and female (on the right). Male mouth parts **(A)** Poor-development of maxillae and mandible, bristle-shaped. Female mouth parts **(B)** Labellum **(C)** Maxillae (lacinia), **(D)** Mandible **(E)** Labrum **(F)** Hypopharynx.

Culicoides adults bear the following parts:

- Head morphology: composed of compound eyes, antennae, maxillary palpus and mouth apparatus (Fig. 4A,B).
- Thorax morphology: divided in three parts, prothorax, mesothorax and metathorax (Fig. 4C). Each part bearing a pair of legs.
- Abdomen morphology: composed of 10 segments and genital appendices (Fig.4D)

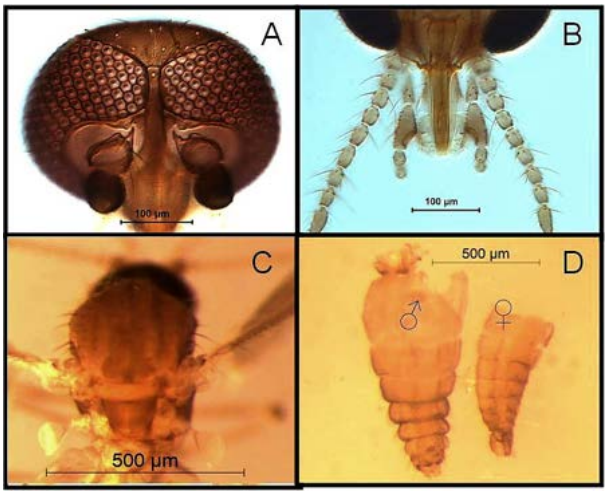


Fig. 4. *Culicoides* sp.

(A) General aspect of the head

(B) Mouth parts, palpus and antennae

(C) Thorax

(D) Genitalia

- Palps and sensilla coeloconica morphology (Fig. 5).

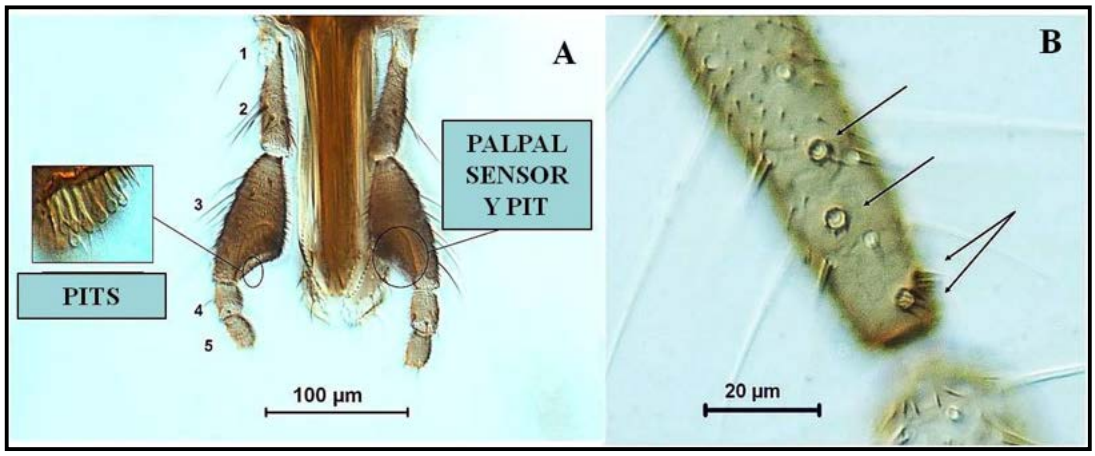


Fig. 5. *Culicoides pictipennis* ♀: **(A)** Maxillary palpus segments (1-5), and pits in detail **(B)** Typical morphology of sensilla coeloconica.

- Wings pattern, veins and cells nomenclature (Fig. 6).

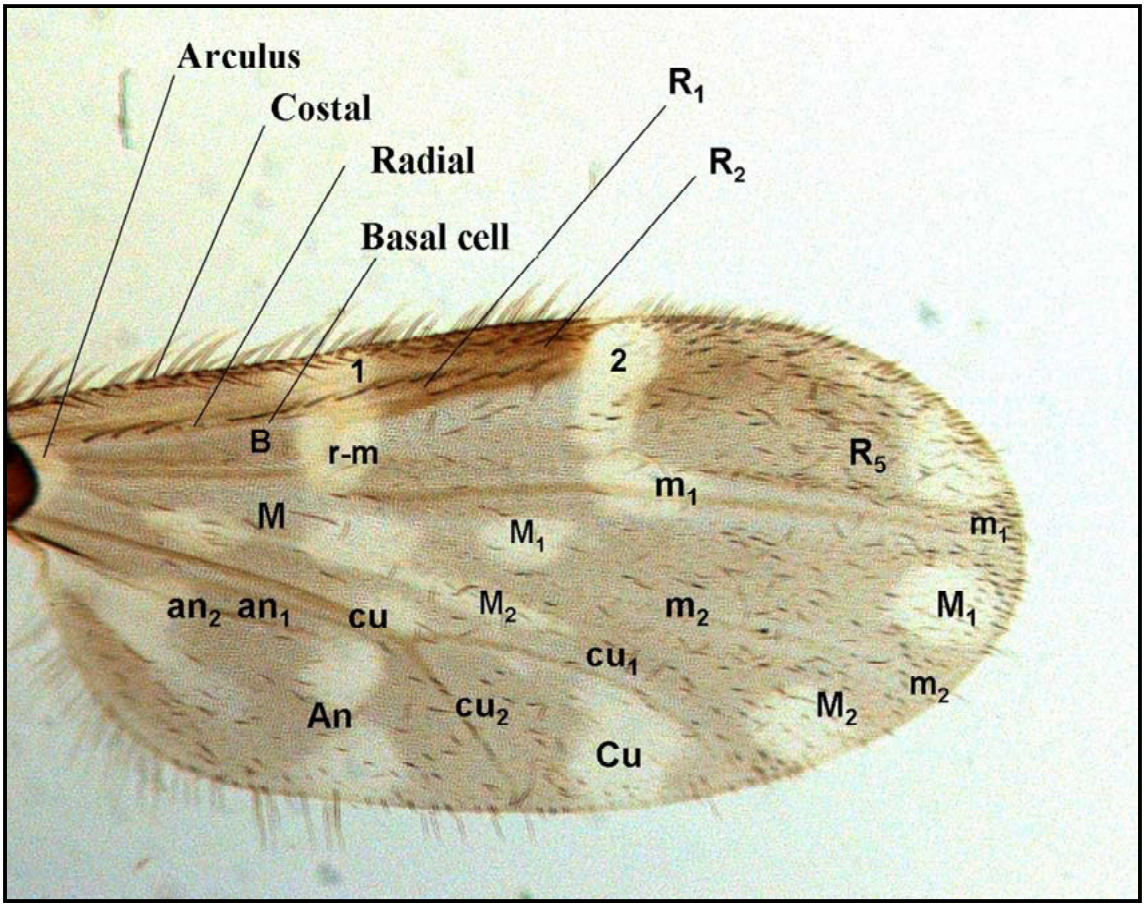


Fig. 6. *Culicoides festivipennis* ♀; wing morphology.

-Cells: R_1: 1º radial; R_2: 2º radial; R_5: 5º radial; M: Medial; M_1: Medial cell 1º; M_2: Medial cell 2º; Cu: Cubital cell; An: Anal; 1: First costal spot; 2: Second costal spot; B: Basal cell.
-Venation: m_1, m_2, cu, cu1, cu_2, an, an_1, an_2, r-m, costal vein, radial vein, arculus.

- Male (♂) and female genitalia (♀) morphology (Fig. 7).

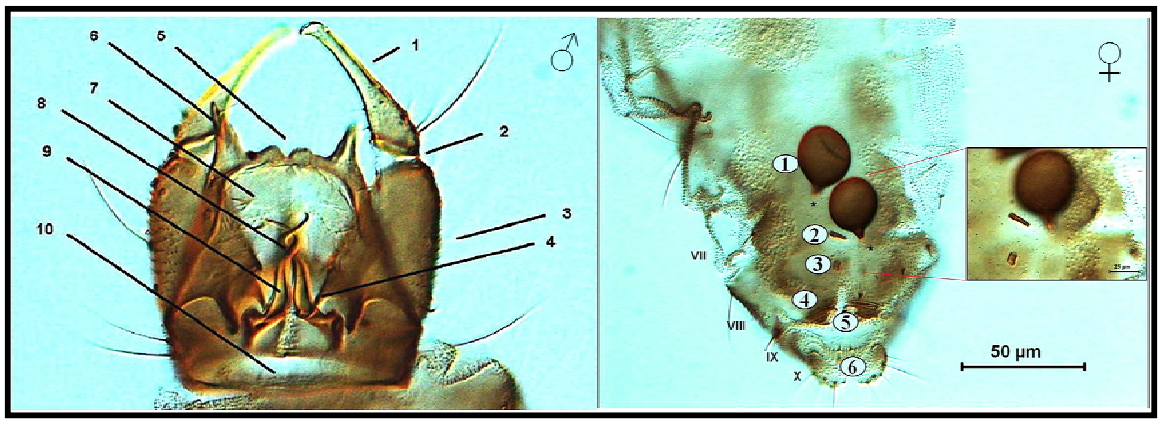


Fig. 7. *Culicoides kibunensis* ♂; genitalia: **(1)** Ditistyle **(2)** Joint **(3)** Basistyle **(4)** Ventral root **(5)** Lobes and caudomedian excavation **(6)** Apicolateral processes **(7)** Cercus **(8)** Aedeagus (blurred) **(9)** Parameres **(10)** Ninth sternite. *Culicoides pallidicornis* ♀; genitalia: **(1)** Spermathecae **(2)** Rudimentary spermatheca **(3)** Anal sclerite **(4)** Chitinous plates **(5)** Genital space between plates **(6)** Cercus. Abdominal segments represented with Roman numerals: (VII-X).

4.2. Immatures

Specific features of Culicoides. The four larval instars are slender, hairless and vermiform without prolegs in the thorax, in contrast to the closely related genus *Forcipomyia* (Fig. 8). The head capsule is prognathuous without conspicuous apodemes and all with setae simple. The head colour is yellowish to brownish with translucent-whitish body. Usually the presence of patches on thoracic segments of subcutaneous pigment is species characteristic. Abdomen with normal segments, not secondly divided. The most notorious structure in the head is the epipharynx, which is used to crush food together with the hypopharynx (de Meillon and Wirth, 1991; Borkent and Spinelli, 2007). The forms of these structures are different among species groups, depending on the feeding pattern. Chaker (1983) classified two types: those with pharyngeal chewing mouthparts and those with sucking-filtering apparatus type. Pupae are typically Ceratopogonid shape, with prothoracic respiratory horns usually elongated bearing a characteristic series of lateral and terminal spiracular openings. Chaetotaxy, tubercles and setation distribution are useful to Ceratopogonid group identification and sometimes specific to species level.

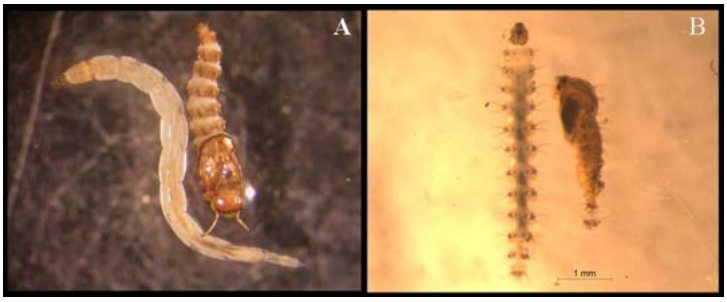


Fig. 8. Immature stages

(A) Larvae and pupae of *C. sonorensis* (courtesy of Prof. Dr. B.A. Mullens)

(B) Larvae and pupae of *Forcipomyia* sp.

5. DISTRIBUTION

5.1. World distribution

The Ceratopogonidae family comprises a total of 6,358 species (6,089 extant and 269 fossil species) and 134 genera (110 extant and 24 fossil genera) (Borkent, 2012b). Biting midges of the genus *Culicoides* are a richer group species, with more than 1,322 species distributed worldwide, ranging from the Tropics to the tundra and from sea level to 4,000 m, with the exception of the Antarctica region (Mellor et al. 2000).

- World distribution *Culicoides* species by ecozones:

- Nearctic Region: around 150 species have been recorded (Borkent and Grogan, 2009).
- Neotropical Region: around 290 species (Borkent and Spinelli, 2007; Borkent, pers. comm.).
- Afrotropical Region: 112 species in South Africa (Meiswinkel et al. 2004).
- Australasia Region: around 265 species (Dyce et al. 2007).
- Southeast Asia: 168 species (Wirth and Hubert, 1989).
- Palaearctic Region: more than 300 species could be present, of these Europe contains 129 species (Szadziewski and Borkent, 2004)

Despite this great species richness, it would be reasonable to estimate that there are at least 15,000 morphologically distinct species on the planet, as many species remain undescribed, many areas are still virtually uncollected and the majority of the studies have been focused on places with permanent livestock (Borkent, 2005). Below is a map with the species distribution in worldwide ecozones (Fig. 9).

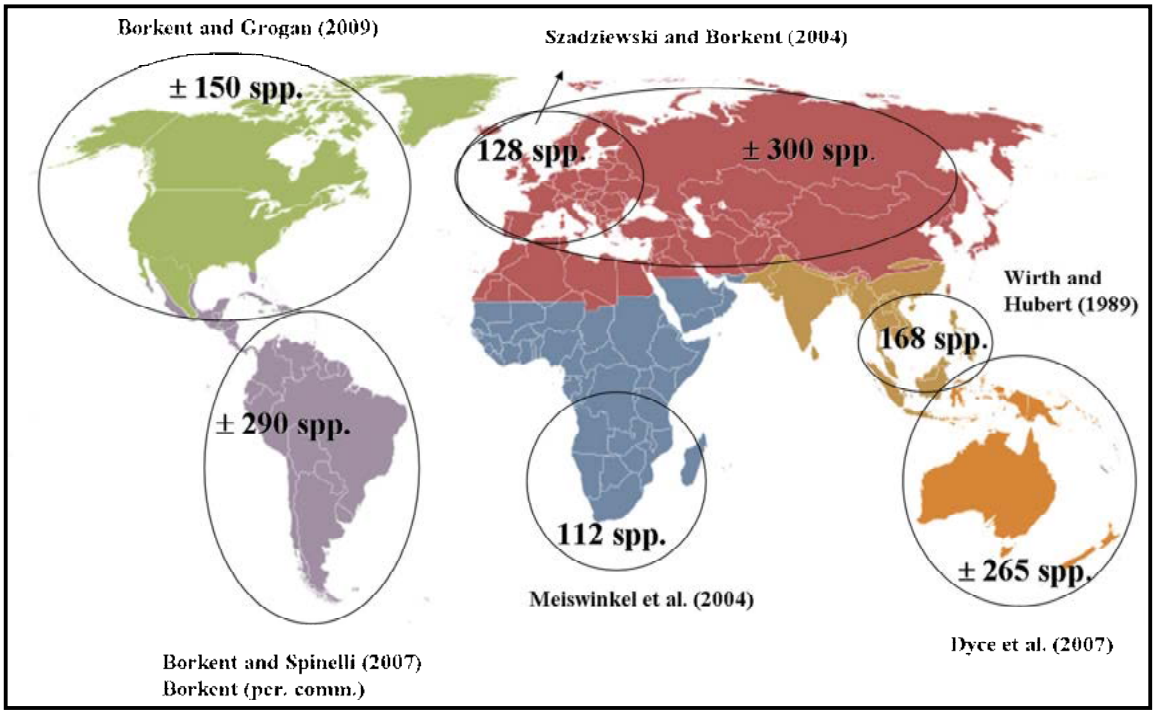


Fig. 9. Total number of *Culicoides* species in world´s ecozones.

The *Culicoides* species recorded from certain European countries are represented in Figure 10. However, the total number of species is probably not exhaustive and underestimated in some regions. First, because the majority of the studies are focused on the most important species or species complex candidates of pathogen transmission (veterinary interest), which usually includes: *C. imicola* in South Europe and Obsoletus and Pulicaris complex in Central-North Europe. Second, because monitoring programs usually only include few localities of study, with short term-monitoring, plus an inefficient effort in species identification and also a lack of sampling in natural or sub-farming regions, which generally increase the number of collected species. For this reason, Figure 10 should be interpreted as a compilation and approach that compiles the most relevant contributions in Europe (updated January 2013).

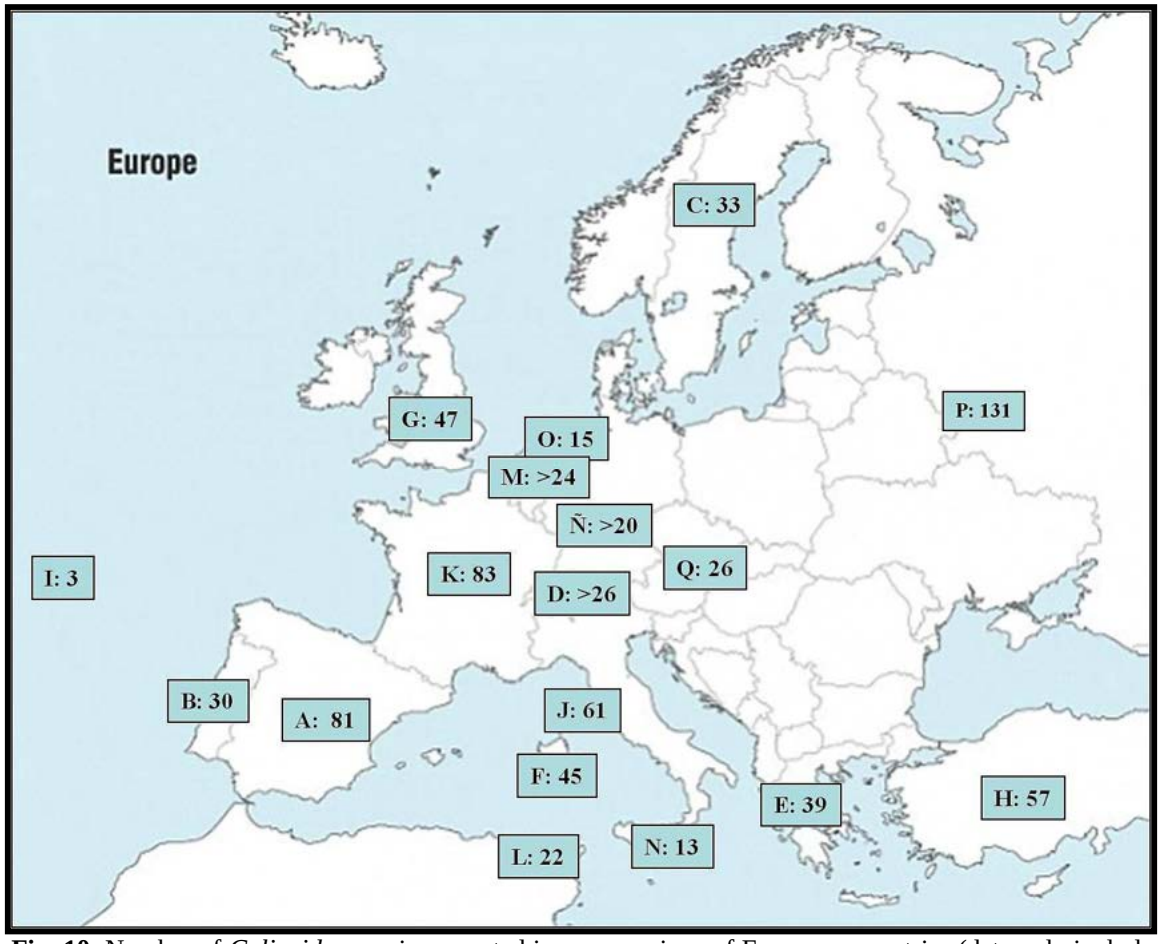


Fig. 10. Number of *Culicoides* species reported in some regions of European countries (data only include faunistic checklist from 2006 to 2013). Letters correspond with the following countries and references **(A)** Spain (Alarcón-Elbal and Lucientes 2012); **(B)** Portugal (Ramilo et al. 2012); **(C)** Sweden (Nielsen et al. 2010); **(D)** Switzerland (Casati et al. 2009; Kaufmann et al. 2012); **(E)** Greece (Patakakis et al. 2009); **(F)** Sardinia (Foxi et al. 2011); **(G)** United Kingdom (*Culicoides*.net, 2012); **(H)** Turkey (Dik et al. 2006); **(I)** Azores (Ramilo et al. 2012); **(J)** Corsica (Delécolle et al. 2005); **(K)** France (Venail et al. 2012); **(L)** Tunisia (Hammami et al. 2008; **(M)** Belgium (Zimmer et al. 2009; Fassotte et al. 2008); **(N)** Sicily (Guercio et al. 2010); **(Ñ)** Germany (Kiel et al. 2009); **(O)** Netherlands (Takken et al. 2008); **(P)** Russia and adjacent lands (Glukhova, 2005); **(Q)** Austria (Anderle et al. 2008).

5.2. Spanish mainland distribution

There are 81 species of *Culicoides* reported in Spain to date, divided in 9 subgenera, a miscellaneous group and a *nomen dubium* (Alarcón-Elbal and Lucientes, 2012) (Fig. 11A).

First accounts of *Culicoides* in Spain date back to the 1990s with Gabriel Strobl. Since then, several authors have contributed to the increase of knowledge of these biting midges in the Iberian Peninsula region. An updated catalogue of the species of this genus in our Country as well as a critical review of the literature, clarifying chronological aspects of the last century publications research can be consulted in Alarcón-Elbal and Lucientes (2012).

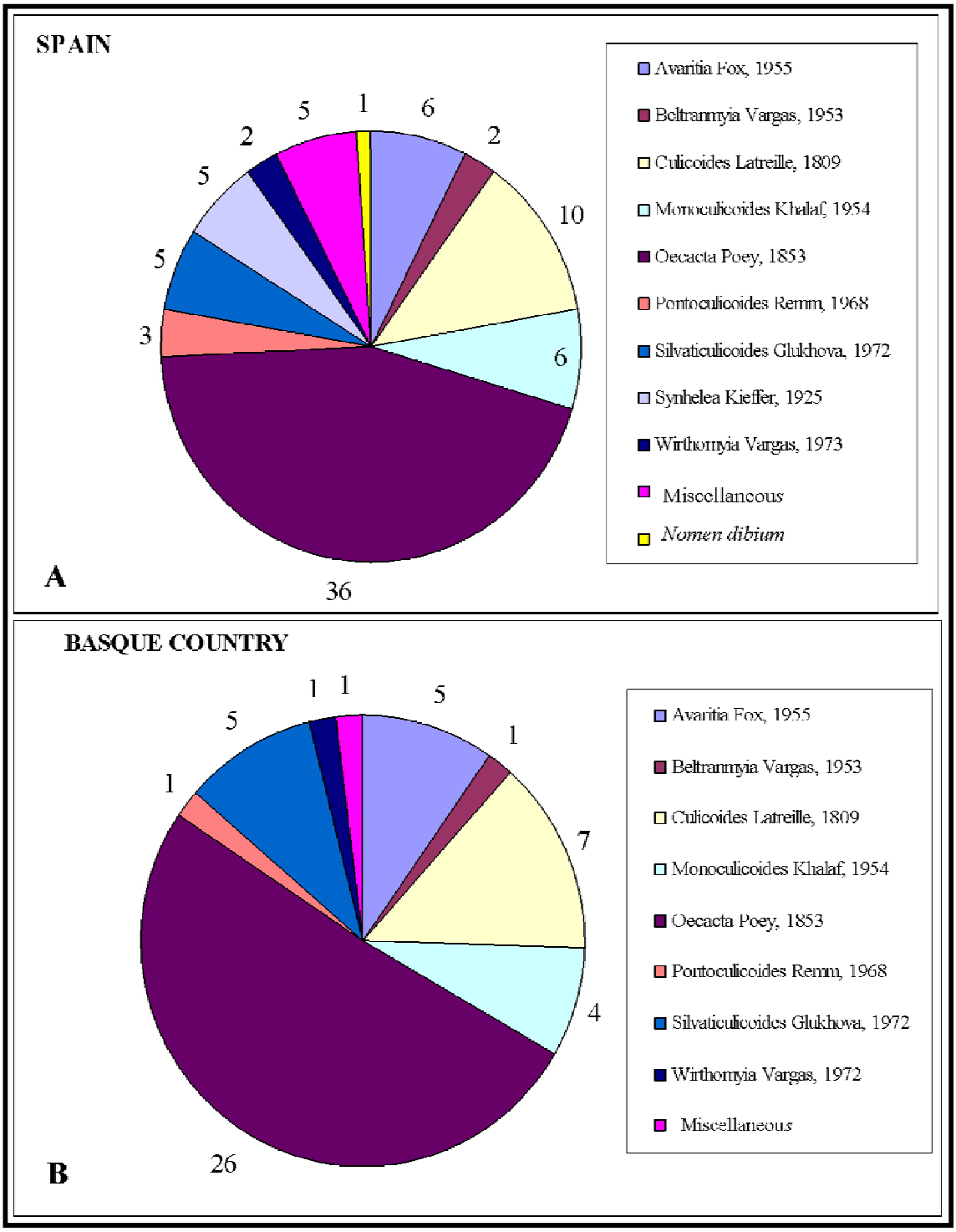


Fig. 11. Distribution of subgenera *Culicoides* in **(A)** Spain **(B)** Basque Country (González and Goldarazena, 2013a).

However, in Spain only certain regions have been studied rigorously in recent years. For example, Catalonia (the northeast part of Spain) is well-studied, recording up to 53 *Culicoides* species in that area (Talavera et al. 2011). Several works in Catalonia about diversity and distribution of *Culicoides* species have been published, enabling an important increase of knowledge for this region. Some examples are the contributions of

Sarto i Monteys and Saiz-Ardanaz (2003), Sarto i Monteys et al. (2009) and Talavera et al. (2011).

Similarly, in northern Spain the biodiversity of the genus *Culicoides* is poorly known, except for some species cited in Navarre by Gil Collado and Sahuquillo Herráiz (1983). Currently, the most relevant contributions in that area belong to Goldarazena et al. (2008), Romón et al. (2012), González and Goldarazena (2011) and González et al. (2013a) who carried out an important work of this group in faunistic terms in the Basque Country, accounting for 52 *Culicoides* species divided into eight subgenera and a miscellaneous group (Fig. 11B). Moreover, *Culicoides* species distribution and phenology of the most important public health species groups from Andalusia (southern Spain) is reported by Pérez et al. (2012). Unfortunately, several regions in Spain remain without intensive studies. Faunistic inventories are important to list the biodiversity, to analyze the possible spread of species into new territories and to study the species communities in relation to the environment.

Nevertheless, BTV outbreaks have led to the development of some surveillance programs such as: Spanish National Entomological Surveillance Identification Program in 2004, which have allowed the *Culicoides* monitoring of more than 200 points on the Spanish mainland and adjacent islands (Lucientes et al. 2008). This program enabled the discovery of the first *Culicoides* distribution data in the whole of Spain. The immense number of captures and implicit difficulties for *Culicoides* identification, made necessary the exclusively focus on certain species groups such as Obsoletus group, Pulicaris group and *C. imicola* species, dismissing the remaining species.

6. *CULICOIDES* BIOLOGY

6.1. Life cycle and factors implicated

Culicoides species are holometabolous insects, whose lifespan comprises 4 stages: egg, larvae (I, II, III, VI), pupa and adult.

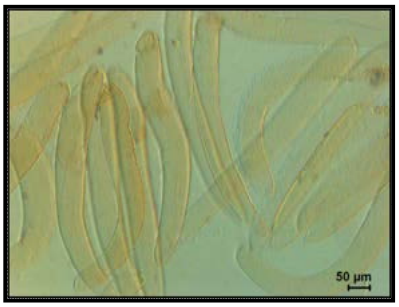


Fig. 12. *Culicoides nubeculosus* eggs

Culicoides females lay eggs in those places where organic matter and moisture are available to allow the development of the larvae. The availability of humidity is one of the most important essential requirements together with suitable breeding sites.

The eggs are large (350-500 µm in length, 65-80 µm in width) and light-coloured at the beginning, then the eggs quickly become darker with air contact (Fig. 12). The number of oviposited eggs is extremely variable depending on the species and biotic factors, ranging from 10 to 675 (Chaker, 1983). Females lay their eggs either in lines or more commonly in small batches. *Culicoides circumscriptus* lays a mean of 250 eggs (Becker, 1961), whereas *C. pulicaris* oviposites an average of 93 eggs (Parker, 1950). The time of hatching tends to be shorter, generally few hours. It is strongly conditioned by the external conditions.

Larvae I size is no more than 0.5 mm length, and after three moults, it acquires the larval stage IV with a length near one centimetre in the largest *Culicoides* species. Larvae bear an apneustic breathing system. Although some terrestrial *Culicoides* species crawl on the soil, others are good swimmers, making wave-snake-movements "serpentine" through the semi-solid media where they inhabit (EFSA, 2007). Interestingly, some species (e.g. *Avaritia* subgenus) in general prefer non flooded soils. *Culicoides* larvae horizontal-vertical distribution and feeding habits are described in **Chapter: 3.**

The lengthening of the larval cycle varies greatly among *Culicoides* species, depending mainly on temperature and the number of daylight hours, varying from a few days to several months or also years in Arctic latitudes. It is thought that larvae go into

the diapause stage at the end of the warm season, with the falling temperatures and daylight reduction, and in the following spring with warmer temperatures and more daylight hours, they continue their development. Curiously, certain studies carried out recently did not report any larvae in the winter season in favourable breeding sites (Uslu and Dik, 2005, 2011).

Pupae stage is a non-feeding stage, usually with a shorter duration (few hours).

Finally, winged adults emerge, and depending on species and outer temperatures the longevity takes between a few weeks (usually) to several months (rarely) (Mellor et al. 2000). Similarly, the majority of longevity studies have been performed in laboratory conditions, for instance *C. obsoletus* was kept alive for up to 90 days, while adult midges reach in general an adult lifespan of only one month (Nevill, 1971; Boorman, 1991). These results most likely vary in wild conditions where climatic factors and temperature experiment sudden changes affecting the correct *Culicoides* development. Optimal range of temperatures is specific to each *Culicoides* species, in general temperatures below 8-10 ºC and up to 35-40 ºC are not favourable.

One of the most critical questions is: where and in what stage(s) do *Culicoides* species live during the winter? In **Chapter 3,** three main hypotheses are formulated that are briefly explained here:

i) adults survive in winter: light CDC traps collections in central and north Europe collected small proportion of a active adults or a new emergence of midges from suitable nearby breeding sites every month throughout the entire winter period. The presence of parous and/or gravid females during winter may herald the onset of bluetongue disease and indicates the overwintering ability of adult midges (Losson et al. 2007; Muijskens, 2008).

ii) eggs and larval stages overwinter: the tiny size of *Culicoides* eggs do not allow field observation in their natural hatching sites, even though this behaviour pattern has been observed in many groups of arthropods. Larval development continues during the winter (at least a few specimens emerged) according to the collection of some nulliparous specimens every month throughout the winter period by European

researchers (EFSA, 2007). Similarly, Dzhafarov (1964) collected immature *C. pulicaris* instars larvae under the surface of the ice.

iii) *Culicoides* midges arrive from other regions (perhaps warmer areas): in some tropical and sub-tropical areas the life cycle of *Culicoides* species continues in non-stop generations year on year. Soft climate and frequently raining periods are likely the responsible for this strategy. This theory is consistent with the fact that *Culicoides* midges seem to have two types of flight, short distance flights (1-2 km) and long distance flights (up to 700 km), in which midges are passively carried by wind because of their small size (Sellers, 1992; Wilson and Mellor, 2008).

6.2. Breeding sites

A great variety of micro-habitats are used by the *Culicoides* species to lay their eggs, including a) lakes, b) mud near ponds and pools with or without vegetation with organic matter, c) fallen leaves, d) rotten plants and fungi, e) livestock dung, f) water reservoirs contaminated with faeces, g) sewer systems, h) puddles, i) swamps, j) drainage tunnels, k) marshes, l) tree holes, m) riversides, n) mud alongside streams, o) different blends of silage residues, p) grasslands, q) standing water, r) dripping water, s) different types of wet soils rich in organic matter, t) faeces-contaminated straw, u) runoff areas and v) different types of manure among others (Hill, 1947; Jones, 1961; Kettle and Lawson, 1953; Kremer, 1965; Jamnback and Wirth, 1963; Weinburgh and Pratt, 1962; Jamnback, 1965; Dzhafarov, 1964, 1976; Braverman et al. 1974; Trukhan, 1975; Mirzaeva et al. 1976; Mellor and Pitzolis, 1979; Mathieu, 2005; Uslu and Dik, 2007, 2010; Foxi and Delrio, 2010; Zimmer et al. 2008, 2010, 2012; Ninio et al. 2011; González et al. 2013b; Harrup et al. 2013; Thompson et al. 2013).

In general, the species included in subgenus *Avaritia* are involved in the transmission of different economical disease-losses, such as bluetongue virus (BTV), and thus they are commonly encountered linked to vertebrate presence. The particular association between coprophilic *Culicoides* and livestock has been reported in potential *Culicoides* vectors in Africa, Asia, Australia, America and Europe. In such cases, *Culicoides* species were observed breeding exclusively on dung pats or in different substrates blended with manure (mainly from livestock but also from chicken) as the

principal constituent. It is thought that characteristics of the larval stages of *Avaritia*, (they do not show "serpentine" swimming movements in contrast to the majority of the remaining *Culicoides* species), and from the pupae (they cannot float and usually drown when submerged in water) may be an adaptation to life in semi-solid media (coprophilic micro-habitats) where there are a elevated levels of moisture for egg laying (EFSA, 2007).

Chapter: 3 shows an extensive description of the developmental sites of some potential vectors of BTV *Culicoides* species and also comments upon *Culicoides* species with unknown veterinary importance.

6.3. Swarming and mating behaviour

Swarming flight and mating processes of several *Culicoides* species were reviewed by Downes (1955) whose extensive observations and experiments of several British species are still of enormous importance today. Many detailed facets of the mating process in many *Culicoides* species are still obscure. Indeed, much more information is available for *Culicoides brevitarsis* and *Culicoides variipennis* (Zimmerman et al. 1982), *C. impunctatus* (Blackwell et al. 1994a), *C. melleus* (Linley and Adams, 1972; Linley and Carlson, 1978, 1982, 1983) and *C. nubeculosus* (Downes, 1955, Mordue and Mordue, 2003). Currently, a relative ignorance exists about the mating progress of some species of veterinary interest such as the Obsoletus and Pulicaris groups.

- Types of courtship in *Culicoides*

i) The most widespread courtship behaviour among *Culicoides* species (*C. nubeculosus*, *C. variipennis*, *C. riethi*, *C. punctatus*, *C. pulicaris*, *C. impunctatus*, *C. grisescens* and *C. pallidicornis*) occurs with the formation of a great swarm of male midges near the potential breeding sites. Once female flies go into a swarm of males, they rapidly intercepte (species-specific recognition) and copulation take place during descent to the ground and they usually remain in copula during a short period of time (1-3 min), (Downes, 1955; Zimmerman et al. 1982). Most species mate only once but some species can also mate repeatedly and stored sperm would last for up to three egg batches. It has

been hypothesised, that the female could transfer a pheromone from the production sites of the abdomen to the wings, fluttering its wings and aiding and spreading pheromone into the air.

- The mating of *C. nubeculosus* could comprise both types of courtship (type i and iii). Kremer et al. (1979) reported the existence of an unknown pheromone released by the females to attract males and stimulate copulation. Mordue et al. (2002) described this volatile pheromone as a simple saturated hydrocarbon named *n*-heptadecane.
- In turn, females of *C. impunctatus* appear to produce an aggregation or recruitment pheromone to attract individuals of the same sex (Blackwell et al. 1994a).

ii) Copulation of *C. melleus,* a widely distributed species in the United States, in nature almost certainly occurs on the ground without male swarming flight. *Culicoides melleus* male and female meet through olfactory and visual stimuli and subsequently copulation takes part (Linley and Adams, 1972; Linley and Carlson, 1978, 1982). In the laboratory, different types of experiments were performed with successful mating observations (Fig. 13). Linley and Carlson (1978) reported the possible existence of a contact pheromone which stimulates the male copulation attempt. This could be attributed to the attractant-stimulant activity of the hydrocarbon fraction from hexane extracts of female *C. melleus*, which corresponded to the identification of several methyl-substituted alkenes as candidates (2-methyl-docosane, 8-methyl-docosane, 9-methyl-tricosane, 10-methyl-docosane).

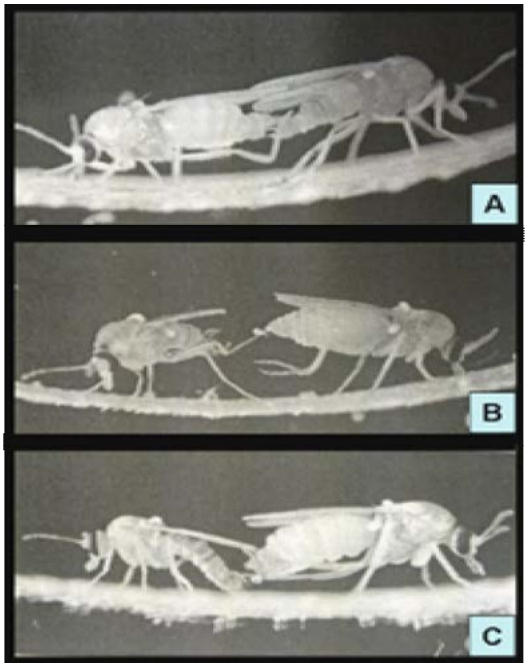


Fig. 13. *Culicoides melleus* major behavioural episodes during copulation (adapted from Linley and Adams, 1972)

(A) Female using hind tarsi to complete separation

(B) The instant of separation; male with partly counter-rotated terminalia, female with almost completely extracted but still adherent spermatophore

(C) Female about to extract spermatophore with grooming organs (tibial combs) of the hind legs

iii) In a reduce number of species the males do not swarm, both sexes are attracted by the same mammalian host, whereby males land on the host and copulate with females before or while they feed on blood (e.g. *C. nubeculosus* and other non-Palearctic species such as *C. utahensis* and *C. sinanoensis*). This mating behaviour is known as substrate-based mating system (Yuval, 2006). This is also the case of *C. puncticollis*, a stenogamous species present in some regions of the Iberian Peninsula (Glukhova and Dubrovskaia, 1974; Sarto i Monteys et al. 2009).

- Characteristics of male swarms

Many midges species form male swarms (leks) over markers on the ground into which virgin female midges fly prior to mating (Mordue and Mordue, 2003).

In general, *Culicoides* spp. swarms are species-specific, usually with spherical-subspherical shaped, consist of males, with some exceptions of females incursions, with different number of individuals (ranging from a few to various dozens, but even of thousands as recorded in *C. impunctatus* and *C. variipennis*). *Culicoides* midges are in continuous movement within the swarm, usually with upwards and downwards spiralling and/or zig-zag patterns. Males usually face into the wind with antennal setae erect. Swarms usually take place at different heights, ranging from a few centimetres to 3 meters above ground level. Some other differences in their behaviour may be observed depending on each species, type of swarm and other climatic and unknown factors (Downes, 1955; Zimmerman et al. 1982). Mating occurs typically in flight but also in nearby vegetation at different days and time (preferably before sunset but also at dawn). Parker (1949) noted for first time the concept “swarm-markers” in *Culicoides*. Marker swarms are areas of more or less horizontally contrasted light and dark patches which act as visual cues objects, e.g. soil colours, brightness, vegetation, intersecting paths and others, where swarms are always formed and each individual responded separately. Swarms may occasionally form near or above the vertebrate host (Fig. 14A,B).

Downes (1955) suggested that “males of *C. nubeculosus* perceived the presence of a female among them by means of their antennae, which would function as auditory organs, sensitive to the sound of a flying female, when the long antennal setae were erected. However, mating between non flying males and females, without sound production or auditory response, was also recorded”.

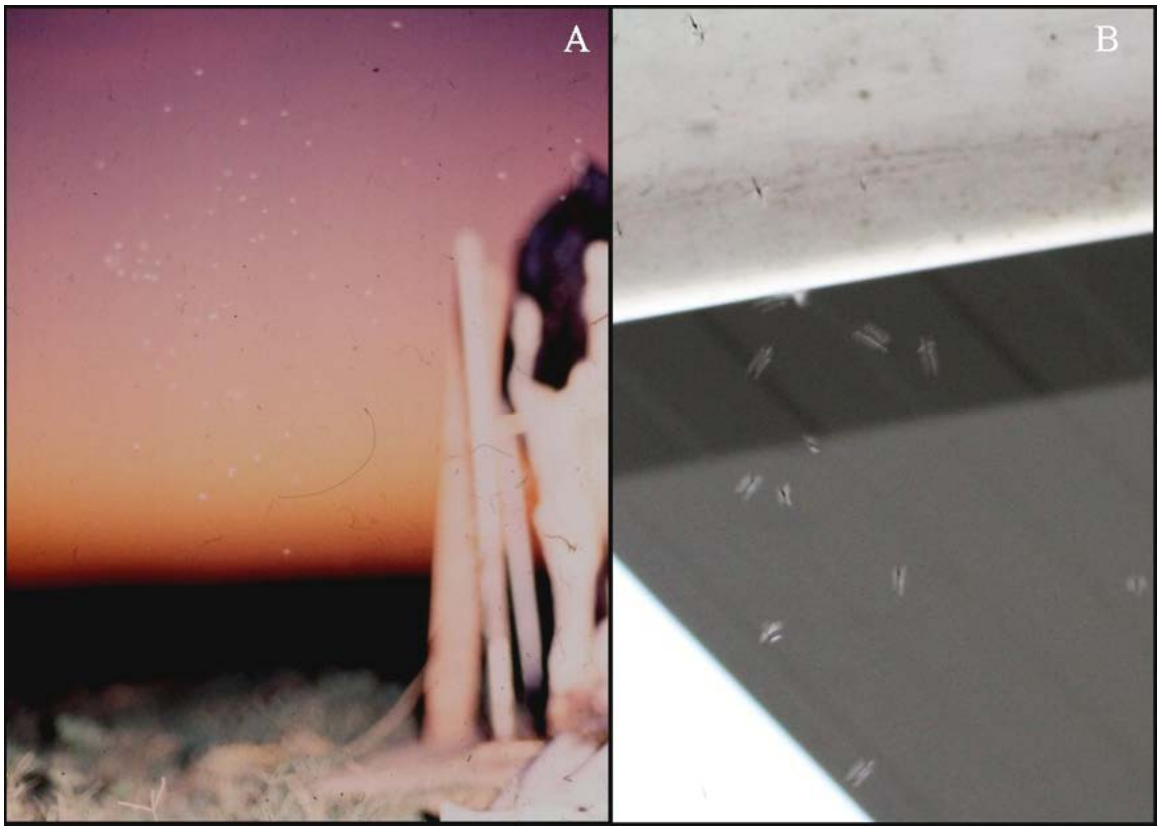


Fig. 14. Swarms of *Culicoides* **(A)** a male swarm of *C. sonorenis* downwind of a calf (Courtesy of Dr. A. Gerry) **(B)** Detail of male *C. punctatus* in spiralling movements near to a silo (farm of Elguea, Spain).

6.4. Feeding pattern

Culicoides midges are among the most harmful haematophagous insects. Studies on host feeding preferences have demonstrated that *Culicoides* females feed primarily on mammals and birds (Santiago-Alarcon et al. 2012). Although most species require a blood meal for egg formation, there are a few species such as *C. circumscriptus* and *C. impunctatus* that are autogenous for their first batch of eggs (Boorman and Goddard, 1970; Blackwell et al. 1992). Females feed principally on blood, but also on sugar and nectar from flowers. They have been observed feeding directly from aphids, to obtain additional nutrients necessary to provide longevity and flight activity. In contrast, males feed exclusively on sugar sources such as plant nectar. Thus, their mouth parts are badly-developed, not capable of sucking-blood (Chaker, 1983).

- Analysis methods of feeding preferences

During the middle of the last century *Culicoides* feeding preferences were recorded based on mere field observations and microscope observations of the blood-meals (erythrocytes in mammals are anucleate whereas other vertebrates have nuclei). In 1970s, the precipitin tests used with *Culicoides* blood-meals enabled the first identification of the blood meal origin (Nevill and Anderson, 1972). Subsequently, in the 1980s enzyme-linked immunosorbent assay (ELISA) became popular for avian and mammalian antibodies identification (Blackwell et al. 1994b, 1995). Over the last decade, DNA-based techniques have grown in popularity as a means to identity the sources of blood-meals for medically important diseases (Townzen et al. 2008). Blood-meals are analyzed by PCR amplification followed by comparing gene sequence with online available database. In some cases, a previous step is performed with species-specific primers on the most common host in the sampling area. Different markers are used: Prepronociceptin gene (PNOC), and more commonly mtDNA cytochrome oxidase subunit I (COI) gene, and mitochondrial cytochrome b (Cyt b), (Townzen et al. 2008 and references therein).

Not only molecular tools give clues about the feeding habits of *Culicoides* species, animal-baited traps and/or direct aspiration on animal's skin also allow the

computation of the conditional host preference of dominant *Culicoides* species and the preferred landing sites. However, host-baited traps also implicate certain problems.

Viennet et al. (2012) suggested that probably the presence of specimens (females collected from a mare were engorged on sheep blood) on the sticky covers suggested that these traps may act in part as interception traps, and large animals "hide" smaller hosts.

In addition, some authors have studied the use of certain morphometric parameters as a reliable source with which to characterise bird or mammal feeding preferences. For instance, Braverman et al. (2012) by means of the number of antennomer bearing sensilla coeloconica in addition with the size of the third palpal segment and values of the antennal ratio recorded of the possible host habits of several *Culicoides* species. In general, authors indicated that bird feeders possess a significantly larger third palpal segment and that the values of the antennal ratio in probable feeders on birds were significantly higher than those in probable mammal feeders. Other aspects such as bulb-shaped sensilla in the pit of the third palpal segment and presence of basal flagellomeres with sensilla trichodea seem to be unreliable for determining host groups. In contrast, Isber et al. (2013) focus their study on the different morphological sensillum types (s.). They found that the number of short blunt-tipped s. trichodea, s. coeloconica, and s. basiconica are significantly higher in the ornithophilic *Culicoides* species compared to mammalophilic, and opportunistic species have an intermediate number of these sensillum types.

- Summary of *Culicoides* feeding preferences

In recent years, several molecular analysis based on blood-meal identification have been carried out in farm settings, in sub-urban areas and also in semi-natural habitats to elucidate the feeding patterns of many *Culicoides* species. The results of six recent molecular studies published about European *Culicoides* species are shown in Table 1.

It is necessary to bear in mind the interpretation of the individual results obtained by each sampling, because *Culicoides* trapping carried out in farms with permanent

livestock tend to be biased to the presence of vertebrate hosts in the study area. Vertebrates such as cattle, sheep, horses and goats are the most common livestock near farming, as a consequence, *Culicoides* midges feed on them and therefore are the most common mammals detected from blood-meal analysis. Unfortunately, *Culicoides* sampling in wild habitats is usually more problematic because of the necessity of a power supply to connect the light traps. Moreover, is not usually possible to quantify the host preferences from these studies, mainly because the available hosts have not been enumerated (Balenghien et al. 2011).

Certain discrepancy among studies has been observed. Overall, some species seem to be biased to mammalophilic and others to ornithophilic feeding habits, likewise *Culicoides* species have broad host preferences, most likely some species have plastic/opportunistic feeding preferences, which are adjusted depending on the availability of host species (Lefévre et al. 2009). Interestingly, two samplings carried out in a sub-urban forest in Germany with walking trails and forest roads, allowed the identification of an important number of *Culicoides* specimens (70% and 84% of the total analyzed blood-meals) contained human blood (Santiago-Alarcon et al. 2012, 2013). Their results indicated that humans can serve as a blood source for dominant *Culicoides* species instead of the normal wild animal hosts in urban areas.

Feeding success depends on host availability, host defensive reactions and host preferences (Viennet et al. 2012). In general, a remarkable percentage of the blood-engorged specimens captured within the subgenus *Avaritia* and subgenus *Culicoides* did not feed exclusively on mammals (including humans) but also on birds (Table 1). However, subgenus *Silvaticulicoides* has been encountered only feeding on mammals. In contrast, *Culicoides* species from the subgenus *Wirthomyia* (*C. reconditus, C. segnis, C. minuttisimus*), subgenus *Beltranmyia* (*C. salinarius* and *C. circumscriptus*) and subgenus *Oecacta* (especially *C. duddingstoni, C. pictipennis, C. kibunensis, C. festivipennis, C. truncorum* and *C. simulator*) are thought to be exclusively avian feeders due to molecular analysis of blood-meals as well as bird-baited traps at canopy level (Martínez de la Puente et al. 2009; Votýpka et al. 2009; Cêrný et al. 2010). Nevertheless, new blood-meal identifications have revealed that some of these ornitophilic species feed also on mammals, including humans (Santiago-Alarcon et al. 2013). Finally members of the subgenus *Monoculicoides* are known to attack livestock animals as

indicated by the blood-meal molecular identifications of *C. parroti* and *C. riethi* (Overgard Nielsen and Christensen, 1975; Gerry et al. 2009).

There is also evidence that different species of *Culicoides* attack different parts of the animal host: back (upper part) is commonly choosen by many species. In contrast other species like *C. obsoletus* did not seem to have preferential sites (Kettle, 1962; Viennet et al. 2012).

Table 1. Blood-meals from subgenus *Avaritia* and subgenus *Culicoides* species.

Subgenus *Avaritia*			Subgenus *Culicoides*		
Species	**Host**	**References**	**Species**	**Host**	**References**
C. obsoletus	**Human**	1,3,5,6	*C. lupicaris*	**Cattle**	1,2,6
	Cattle	1,2,3,4,5,6		**Horse**	1
	Horse	1,2,4,5		**Pig**	1
	Sheep	1,2,3,5	*C. pulicaris*	**Human**	3,5
	Ungulates	5		**Cattle**	3,4,5
	Goat	5		**Horse**	2,5
	Rabbit	1		**Sheep**	3
	Rodent	3,5		**Ungulates**	5
	Bird	3,4		**Rabbit**	1
C. dewulfi	**Human**	5,6		**Bird**	3
	Cattle	1,5	*C. punctatus*	**Human**	3
	Pig	1		**Cattle**	1,2,4,5
	Horse	1,2		**Horse**	1,2,4
	Rabbit	1		**Sheep**	2,3
C. scoticus	**Human**	5,6		**Ungulates**	2,5
	Cattle	1,2,4,5		**Goat**	5
	Pig	1		**Rabbit**	1
	Horse	1,2,5,6		**Rodent**	3
	Sheep	1,2		**Bird**	2,3,4
	Ungulates	4,5	*C. newsteadi*	**Cattle**	2
	Rabbit	1		**Sheep**	3
	Bird	4	*C. deltus*	**Human**	6
C. chiopterus	**Human**	6		**Cattle**	4
	Cattle	1,4,5	*C. impunctatus*	**Horse**	2
	Horse	2		**Sheep**	2
	Ungulates	5	*C. grisescens*	**Cattle**	2
	Bird	4			

References correspond with successful blood-meals identification of **(1)** Ninio et al. (2010); **(2)** Pettersson et al. (2013); **(3)** Calvo et al. (2012); **(4)** Lassen et al. (2011); **(5)** Lassen et al. (2012); **(6)** Santiago-Alarcon et al. (2012). In greyish, avian blood-meals is highlighted.

6.5. Period of flight activity

Circadian cycles of *Culicoides* species are poorly described. It is widely assumed that most *Culicoides* are nocturnal-crepuscular, showing a bimodal pattern of host-seeking activity with peaks at dawn and dusk, and to a lesser extent through the night. Many species show a main activity peak only around sunset (Blackwell, 1997; Mellor et al. 2000; Viennet et al. 2012). During sunset, humidity gradient and temperature decreases, and therefore *Culicoides* midge's activity start. However, on cloudy days with a high gradient of humidity, midges can also fly during the day. The existence of species with diurnal preferences, such as *C. dewulfi* and *C. heliophilus* is reported (Kettle, 1962).

Although THE Obsoletus group has been considered as a nocturnal species, recent studies have shown the existence of clear evidence of diurnal activity for these species. By means of backpack aspirator, several *C. obsoletus* specimens (mainly parous and gravid) were captured on a farm at different hours during the day (Balenghien et al. 2008).

Dik and Ergül (2006) observed that different *Culicoides* species had different flight activities in Turkey. Moreover, Braverman et al. (2003) and Viennet et al. (2012) recorded that peak hour activity in *Culicoides* midges depends on two main factors: geographical position (latitude and longitude) and seasonality (different peaks of activity from month to month) followed by the influence of other parameters such as sunlight, data length, temperature and relative humidity.

Culicoides flight activity has been studied by various authors. For instance, direct aspiration on sheep showed that *C. obsoletus* and *C. parroti* activity were most active at sunset, with the maximum peak 40 minutes before sunset (Gerry et al. 2009). On the other hand, van der Rijt et al. (2008) observed in a tend trap baited with a horse, that the largest number of *C. obsoletus* and *C. pulicaris* were caught at sunset and a few *Culicoides* midges were captured at sunrise and afternoon and very few or none at night. Viennet et al. (2012) rigorously studied circadian host-seeking of Palearctic *Culicoides* species and observed that *C. obsoletus* host-seeking activity occurred mainly around sunset, with slight differences depending on the season.

The diel activity of *Culicoides* biting midges has been also studied using vehicle-mounted traps. The greatest abundance of *Culicoides* was at sunset, with little activity recorded during the two hours before and after sunset. A second peak, usually smaller than at dusk occurred at dawn, but with a bright moon this pattern could reverse, exhibiting a higher activity through the night (Sanders et al. 2012).

6.6. Factors affecting the diel activity of adult *Culicoides*

Weather and climatic conditions (wind speed and direction, air temperature, humidity, lunar phase, brightness, precipitation, light intensity, atmospheric pressure, time) are some of the main meteorological factors with enormous implications on

Culicoides midges flight activity (Mellor et al. 2000). Here are detailed the most relevant ones:

i) Wind: each *Culicoide*s species displays different pattern of flights ranging from 1 to 4 m/s. *Culicoides* species are unable to fly with moderate-strong wind. On average, wind-speeds up to 4 m/s are enough to inhibit flight behaviour (Sanders et al. 2012). In contrast, Viennet et al. (2012) captured a significant number of *Culicoides* with strong wind speeds (5 m/s in average). Wind climatic factors are also responsible for biting behaviour, host-location, short-long distance flights and also mating-swarm formation in certain species (Logan et al. 2010). The role of the wind as long-distance dispersal agent for *Culicoides* is briefly described in **Section 11 (ii)** of this chapter.

ii) Temperature is one of the most extrinsic variables affecting *Culicoides* activity. Warm temperatures with high humidity are optimal for *Culicoides* flight. However, extreme conditions of both factors are not propitious. There was no activity of *Culicoides* with outdoor temperatures below 5º C as no midges were captured in traps. Low temperatures slow down the metabolism and thereby the activity of midges, while the survival of an individual midge increases (Muijskens, 2008). Wittmann et al. (2002) reported that *Culicoides* cannot survive when temperature is consistently lower than 7º C in combination with relative humidity lower than 30% and flight is not observed up to 35-40º C in any species. In general, midge activity is assumed to decrease when outdoor temperatures fall below 10° C (de Koeijer and Elbers, 2007).

Temperature limits are extremely variable for each species. Viennet et al. 2012 observed that flight is initiated at up to 8-10º C, with temperatures below this threshold, only single individuals were collected. In contrast, Muijskens (2008) reported several *Culicoides* spp. collected in traps at outdoor temperatures below 10° C and also reported that midge activity was still observed at outdoor temperatures of 5° C and above.

A reduction in activity was observed above 17-21º C for most *Culicoides* species (Bishop et al. 1995; Sanders et al. 2012). Optimum temperatures for *C. oxystoma* and *C. maculatus*, under laboratory conditions were 25-30º C, (Tsutsui et al. 2011) and similar results have been reported for *C. imicola* (Purse et al. 2006). However, optimal temperatures for *C. brevitarsis* are closer to 20º C and for *C. impunctatus* flight is around 11º C.

iii) Humidity has not so great impact in *Culicoides* numbers and low relative humidity did not seem to inhibit host-seeking activity (Sanders et al. 2012; Viennet et al. 2012). Although *C. chiopterus* was negatively influenced by this parameter, Blackwell (1997) observed a positive correlation with relative humidity and rainfall in *C. impunctatus* flight activity.

iv) Lunar phase: flight activity increases during full-moon periods compared to other moon phases (Barnard and Jones, 1980; Lillie et al. 1987). In the Basque Country, Romón et al. (unpublished data) also observed significantly more captures of several *Culicoides* species in full-moon nights, although *C. obsoletus/C. scoticus* appeared not to be affected by it. Moreover, *Culicoides* species which breed in marine habitats, experimented positive correlated catches with high tidal fluctuations evoked by full-phase moon. Some exceptions were recorded by Bishop et al. (2000), who observed fewer captures in full-moon nights, most likely induced by the competition of light traps with moon brightness. Likewise, Sanders et al. (2012) reported a reduction in trap catches of *C. chiopterus* and *C. dewulfi* in presence of a bright moon or with an increasing lunar phase.

v) Brightness (light intensity): during the day "abnormal outnumbers" of *Culicoides* specimens could also occur. This is attributable to those overcast days with low-light conditions leading *Culicoides* to bite throughout the day and lengthening the duration of *Culicoides* attack hours (EFSA, 2007; Balenghien et al. 2008; Logan et al. 2010; Viennet et al. 2012).

vi) Precipitation: although *C. impunctatus* is active with light rain, it is less so during heavy rain (Logan et al. 2010). Data of field trapping with *Culicoides* midges are usually rejected with heavy rain studies because of the scarce collections. The rainfall season, which is linked to the successful development of high numbers of *Culicoides* larvae is of paramount importance due to the great availability and permanence of semi-aquatic breeding sites (Mellor et al. 2000).

vii) **Sunset time**: Consult section 5.5 (Period of activity).

7. MEDICAL AND VETERINARY IMPORTANCE

7.1. As biting pests

Globally, the most commonly observed impact of *Culicoides* biting midges on public heath occurs through nuisance and annoyance inflicted by the persistence biting of female adults (Carpenter et al. 2013). Certain species have provoked concern for their persistent activity and vast populations in many regions, including northern Europe. *Culicoides* midges act as notorious pests, often creating discomfort for outdoor practices (hunting, campers, hikers, forestry, agriculture) and discouraging tourism during summer months (Hendry, 2011).

i) As pest for humans: Although other integrants of the family Ceratopogonidae such as genera *Leptoconops* and *Forcipomyia* have a worse reputation as a nuisance and nasty biters causing considerable health problems, the lesions caused by the genus *Culicoides* are also painful, itchy and can become infected, resulting in major problems (Krakowski and Ho, 2013). For instance, in Spain, González et al. (2013c) emphasized the episodes occurred in a small village of the Basque Country, where *Leptoconops noei* Clastrier and Coluzzi, 1973 biting midges caused the hospitalization of several citizens troubled by harmful bites betwwen 2004 and 2006. It is likely that more cases occurred in our country but the small size of midges and frequent absence of immediate effect of the bite gives them an unwarranted reputation for invisibility.

Below are detailed the most relevant *Culicoides* species ordered according to their geographical worldwide distribution that are known to disturb humans with their bites (Logan et al. 2010 and references therein).

- *Culicoides impunctatus*. United Kingdom (mainly Western coast of Scotland).

- *Culicoides immaculatus, C. ornatus* and *C. molestus* Australia (Gold Coast).

- *Culicoides furens, C. barbosai, C. hollensis, C. melleus* and *C. mississippiensis*. Coastal areas of United States (Florida beaches).

- *Culicoides floridensis* and *C. tissoti*. Unites States (State of Mississippi).

- *Culicoides furens*. Caribbean islands, eastern seaboard of America from New York in the north to Sao Paulo in Brazil in the south.

- *Culicoides paraensis*. From the northern United States to Argentina.

ii) As pest for livestock: the most relevant problems associated with livestock is a disease known as summer seasonal recurrent dermatitis (SSRD) or seasonal allergic dermatitis or insect bite hypersensibility (IBH) or more commonly named sweet itch.

Different species of *Culicoides* have been reported to be associated with SSRD in horses, in sheep, cattle, goats, cats and also man. However, sheep and cattle are the most common groups of affected animals. In some cases, *Culicoides* bites are companied with other blood-sucking dipterans (Culicidae, Simulidae, Tabanidae, Muscidae) which take part in the biting process. Different *Culicoides* salivary gland antigens have been characterised as the main relevant allergens for IBH in horses. Unfortunately, to date, no satisfactory treatment is available for that disease (Quinn, 1983; Yeruham et al. 1993; Corrêa et al. 2007; Schaffartzik et al. 2012).

iii) Other animal pests: some *Culicoides* species are important avian feeders (Martinez-de la Puente et al. 2009; Cerný et al. 2010). Attacks on insects, amphibians and reptiles are usually attributed to other Ceratopogonidae genera.

7.2. As disease vectors

Three main genera of ceratopogonids (*Culicoides*, *Forcipomyia* and *Leptoconops*) are relevant as vectors of various diseases and parasites of medical importance which can affect animals. The role of *Culicoides* species in the infection of mammals of economic importance, such as domestic and wild ruminants is worthy of mention. Despite the biting nuisance and their role as vectors of internationally important livestock diseases, *Culicoides* have only rarely been implicated as the primary agents of pathogen transmission between humans, except filarial nematodes of Mansonellosis (high prevalence in Latin America and Caribbean and West and Central

Africa) but by far the most important in humans is Oropouche virus (OROV), distributed across a wide geographic range of Central America (Carpenter et al. 2013 and references therein).

Three groups of pathogens and parasites are implicated in worldwide animal diseases:

- Virus: Families Bunyaviridae, Reoviridae and Rhabdoviridae
- Protozoan: Genera *Haemoproteus* and *Leukocytozoon*
- Nematode: Genera *Mansonella* and *Onchocerca*

With regard to Europe, livestock diseases are considered to be of relevant importance due to their economic implications. The following viruses are the three most relevant diseases transmitted by *Culicoides* species in terms of economic/veterinary interest (adapted from Mullen and Durden, 2009).

i) Bluetongue virus (BTV)

- Family: Reoviridae, genus *Orbivirus*, 26 serotypes are now recognized for this virus (Maan et al. 2011).
- Vertebrate host: Cattle, sheep, goat; other domestic and wild animals such as buffaloes, deer, most species of African antelopes and various other Artiodactyla.
- Geographic area: Africa, Middle East, Europe, Japan, Australia, North, Central and South America.
- Vectors: 30 worldwide *Culicoides* species incriminated to various degrees in BTV transmission in four subgenera: *Avaritia*, *Culicoides*, *Monoculicoides* and *Hoffmania* Fox, 1948 (Meiswinkel et al. 2004). Ticks and lice could be also incriminated to a lesser extent.
- Spanish outbreaks: 1956-1960; 2000-today.

ii) African horse sickness (AHS)

- Family: Reoviridae, genus *Orbivirus*, 9 serotypes (Sailleau et al. 2000).
- Vertebrate host: Horses, mules, donkeys, camelids and zebras.
- Geographic area: Africa, Middle East, Europe, Asia.

- Vectors: *Culicoides imicola* and *C. bolitinos.*
- Spanish outbreaks: 1966; 1987-1990. Nowadays, low risk of AHS occurrence in Spain according to de Vos et al. (2012).

iii) Schmallenberg virus (SBV)

- Family: Bunyaviridae, genus *Orthobunyavirus*, single serotype.
- Vertebrate host: Cattle, sheep, goats, bison and possibly alpaca.
- Geographic area: Europe (Germany, Netherlands, Belgium, France, Luxembourg, Italy, Spain, United Kingdom, Switzerland, Ireland, Finland, Denmark, Sweden, Austria, Norway, Poland and Estonia).
- Vectors: *Culicoides obsoletus* group and *Culicoides pulicaris* complex, most likely other species of mosquitoes (Culicidae species).
- Spanish outbreaks: March of 2012.

iv) Others: Global warming and free cross-border transmission could be responsible of the introduction of other "exotic" viruses such as a new outbreak of the African Horse Sickness and others such as Akabane, Epizootic haemorrhagic disease (actually detected in Morocco) and Equine encephalitis (Meiswinkel et al. 2008).

8. BLUETONGUE DISEASE: PATHOLOGY AND ITS ROLE IN WILD ANIMALS

This non-contagious, insect-borne viral infection is inapparent (typically asymptomatic or subclinical) in the vast majority of infected animals but causes fatal disease in a proportion of infected livestock (sheep, goats), deer and wild ruminants. Cattle and species of camelids do not suffer clinical signs with the exception of specific strains of BTV, e.g. serotype 8 currently circulating in Europe, which can induce severe disease.

Clinical signs range from mild to severe and vary not only between species but between breeds and within the flock or herd. Clinical sighs of BT disease are mainly attributable to vascular permeability and include fever, hyperaemia and congestion, facial oedema and haemorrhages, and erosion of the mucous membranes. However in mild cases of the disease, a transitory hyperaemia and slight ocular and nasal discharge may be observed. In very severe cases the tongue may show hyperaemia, become oedematous and protrude from the mouth, or become cyanotic. Hyperaemia may extend to other parts of the body, particularly the coronary band of the hoof, the groin, axilla and perineum. In severe cases there is additionally skeletal and cardiac muscle degeneration. Wool breaks may occur. Sheep may become lame as a result of laminitis and skeletal myopathy (Maclachlan et al. 2009; OIE, 2009) (Fig. 15).

In Spain, sheep are considered to be the most vulnerable species to BTV followed by goats. In fact, ELISA tests (the most common and standards diagnosis test) showed high seroprevalences for bluetongue antibodies in sheep and goat flocks in Spain as in different worldwide regions suffering BT disease (Mayo, 2010).

However, the knowledge of the epidemiology of wild animals in the spreading and persistence of the virus is still scarce. Various studies have demonstrated a high prevalence of antibodies against BTV in different wild ruminant species from central and southern Spain (Ruiz-Fons et al. 2008; Lorca-Oró et al. 2012). In addition, generally the areas where positive wild animals were found coincided with places where BTV outbreaks were detected in livestock (García et al. 2009).

Various conclusions about the prevalence among species and age could be reported. In general, in red deer higher BTV seroprevalences were observed in comparison to fallow deer, roe deer, Spanish ibex and mouflon. Similarly, sub-adult and adult animals had significantly higher prevalence against BTV antibodies than juveniles (García et al. 2009). It is probable that these species could act as reservoir in the transmission and maintenance of the virus.

Fig. 15. Clinical signs of BTV in sheep **(A)** fever, weakness, excessive salivation, apathy, depression **(B)** haemorrhages of the facial mucous membranes **(C)** tongue becomes cyanotic (The photo was obtained from the IAN, Institute of Animal Heath (Pirbright, United Kingdom).

9. SUMMARY OF THE CHRONOLOGICAL EXPANSION OF BLUETONGUE IN SPAIN AND IN THE BASQUE COUNTRY REGION

9.1. Spanish BTV outbreak

- In 1956 an epizootic of BT began in Portugal, and soon spread to Spain. It was the first BT outbreak recorded in Spain. The provinces of Badajoz and Huelva, and thereafter Caceres, Cadiz, Seville, Cordoba, Ciudad Real, and Malaga were affected by the virus (Ortega et al. 1998). The disease remained in the Iberian Peninsula between 1956 to 1960, causing high mortalities in sheep (about 179,000 sheep). The outbreaks were caused by BTV-serotype 2 (Ortega et al. 1999).

- In the second semester of 2000, BTV-2 was detected in the Balearic Islands and also in adjacent Mediterranean countries, thus representing the first incursion of BTV after 40 years of vector-free disease. A total of 4.106 sheep were affected during September and October 2000, in both islands (Miranda et al. 2003).

- In October 2003, a new serotype outbreak (BTV-4) occurred in Corsica and Minorca Islands and in August 2004 BTV-4 appeared for first time in Andalusia and Extremadura. In November, adjoining areas from Portugal with Spain were also affected. A total of 322 cases were declared in Spain: 268 in Andalusia, 50 in Extremadura and four in Ceuta.

- In 2005, the infection reappeared in the communities of Castile La Mancha, Castile and Leon, Extremadura, Andalusia and Madrid, with 89 cases (Allepuz et al. 2010).

- In 2006, the most important event is the arrival of the BTV-8 to Central Europe, with more than 2,079 cases detected in Holland, Luxembourg, Germany and Belgium. Subsequently, serotype-8 reached adjacent countries (Saegerman et al. 2008).

- In 2007, BTV-1 was detected in Cadiz and it quickly spread during the summer to the provinces of Extremadura and Castile-La Mancha. Three months later, a new peak of BTV-1 affected the Basque Country, Navarre and Pyrenees Atlantiques of France (see **9.2. Basque Country BTV outbreak section**). It was precisely in 2007 when the most notorious expansion of the disease ocurred with more than 8,000 outbreaks registered in

different regions of the Iberian Peninsula (RASVE, 2012). Figures 16-17 show the Spanish regions affected by each of the different BTV serotypes during recent years.

- In 2008-2009 the situation in Spain underwent a positive evolution, with a marked reduction in the number of outbreaks (70% reduction in 2008 compared to 2007 and 90% reduction in 2009 compared to 2007). A total of 378 outbreaks occurred in 2009 (377 BTV-1 and one BTV-8).

- In 2010, 91 outbreaks ocurred (80 BTV-1, 10 BTV-8 and one BTV-4). Mainly in Extremadura and Castile, but also in Andalusia and one in Madrid.

- In 2011, 12 outbreaks occurred (nine BTV-1 and three BTV-8). In Extremadura, Andalusia and Castile and Leon.

- In 2012, there were five outbreaks: two BTV-1 (Extremadura) and three BTV-4 (Andalusia). All of them belong to sentinel animals.

Fig. 16. Map with the chronological situation of BTV outbreaks in Spain (2010-2012). Year 2010 (light blue spots), 2011 (red spots) and 2012 (dark blue spots) for the different serotypes outbreaks. Circles indicating serotypes: BTV-1 and triangles BTV-4 (MAGRAMA, 2012).

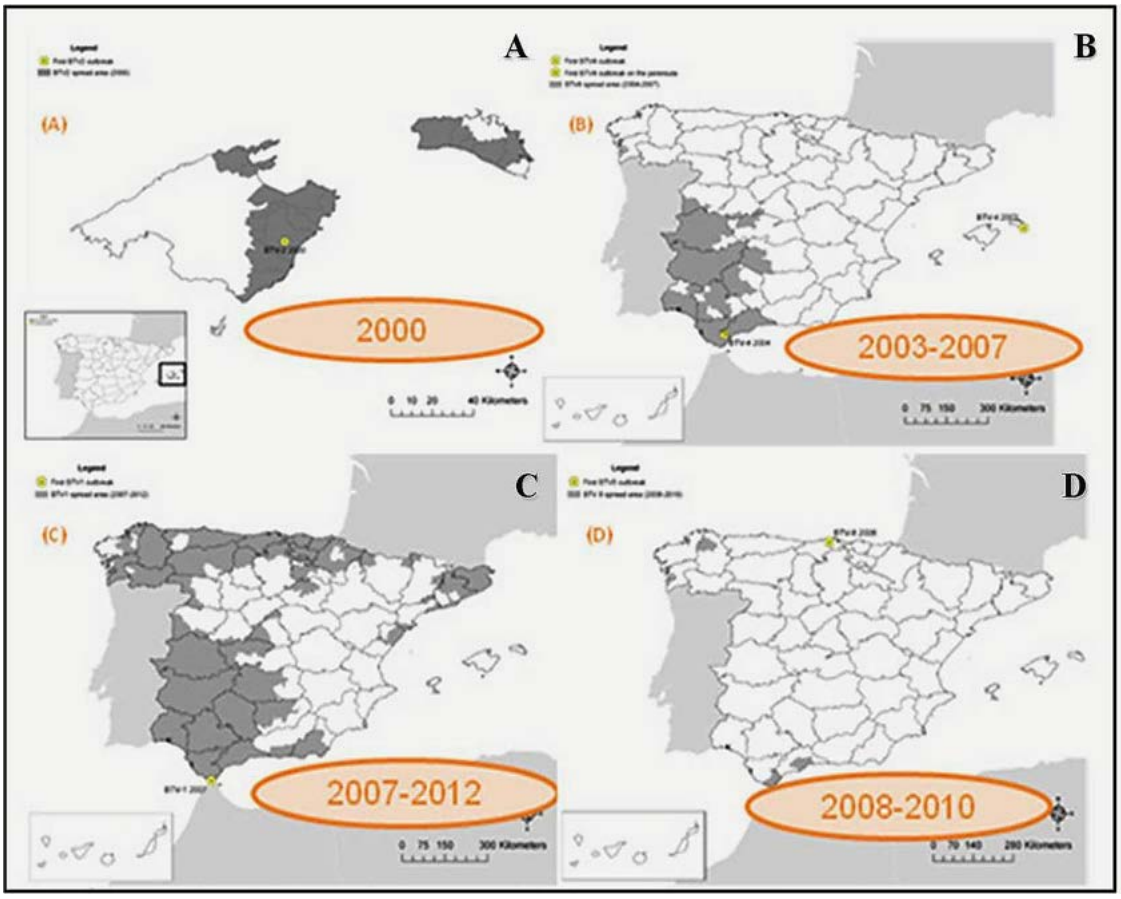


Fig. 17. Spain and adjacent islands affected by BTV serotypes (2000-2012). In greyish, affected regions **(A)** BTV-2 **(B)** BTV-4 **(C)** BTV-1 **(D)** BTV-8 (adapted from Pérez de Diego et al. 2013).

Bluetongue virus control in Spain has been performed with a broad vaccination program for the four detected serotypes from 2000 until today (serotypes 2, 4, 1, 8 in chronological order). Firstly, live-attenuated vaccines were used for BTV serotypes-2, 4, and 2 + 4 (2000-2006), then they were substituted by inactivated vaccines (BTV serotypes 1, 4, and 8, 2006-onwards). Inactivated vaccines are the only one used today (Sánchez-Matamoros et al. 2009). An important number of the outbreaks occurred in recent years correspond to "non-vaccinated experimentation herds" established by the Spanish authorities in susceptible areas of *Culicoides* incursions. During this period (2000-2011), the vaccination program was compulsory but from 31th of July 2011, the Order ARM/3373/2010 was modified by the following ARM/1614/2001, in which the vaccination program becomes voluntary. As of 1 January 2013, the Iberian Peninsula is considered a restricted area fro BTV-1 and a small zone in southern Spain is a restricted area for BTV-4.

9.2. Basque Country BTV outbreak

In November 2007, BTV-1 was detected in Oiartzualdea valley (Oiartzun, province of Gipuzkoa), thus constituting the first outbreak in the Basque Country. A total of 61 outbreaks occurred in 2007 in Gipuzkoa, 39 in sheep flocks, 12 in cattle herds, one in goat flock and nine in mixed farms (sheep, goat and/or cattle in the same farm).

A massive sheep vaccination program began in the three Basque Country provinces in November 2007, which resulted in a considerable reduction, from 19 outbreaks during the winter of 2007 to three in the spring of 2008, all of them in Gipuzkoa. Unfortunately, the vaccination program was initially limited to sheep and subsequently in spring of 2008 it started to be implemented for cattle (not reaching 100% of vaccination), when in August a new emergence of BTV-1 reached Biscay, Alava, and Gipuzkoa a month later (García-Lastra et al. 2012). This new peak left a total of 546 outbreaks (61 in 2007, 478 in 2008 and 7 in 2009) in the Basque Country between 2007-2009, distributed as follows: 324 in cattle herds, 142 in sheep flocks, six in goat flocks and 74 in mixed farms. No more outbreaks have been notified since March 2009. (Fig. 18).

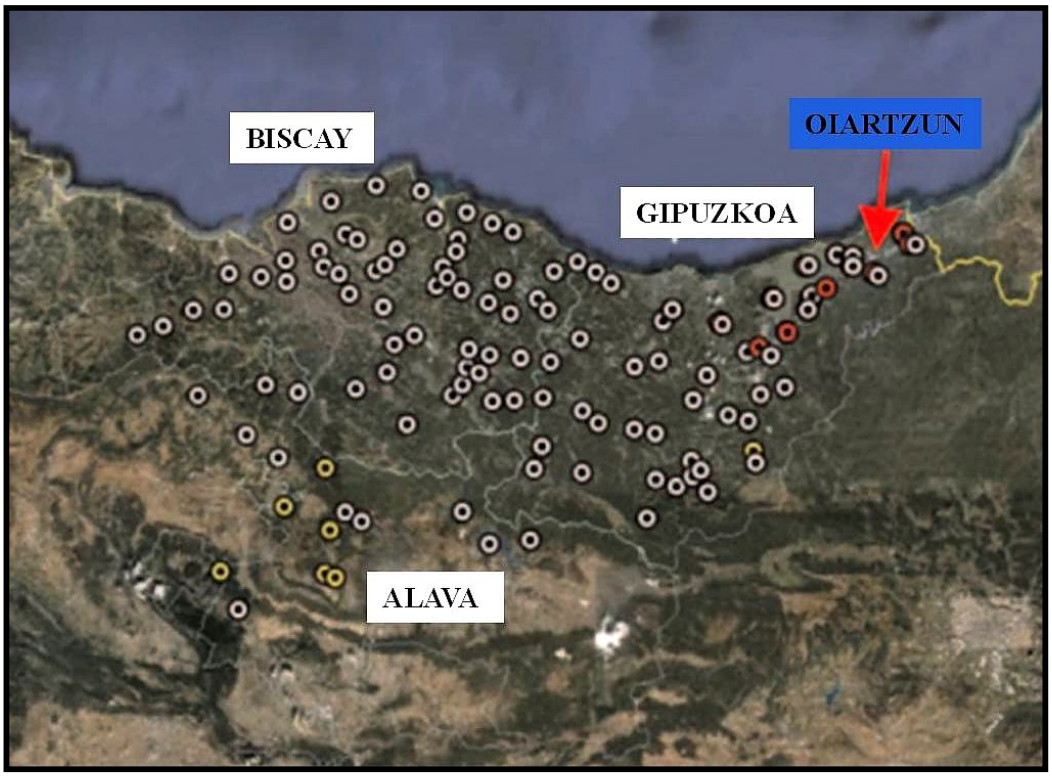


Fig. 18. Basque Country BTV outbreaks (2007-2009). Circle marks show the 2007 (red), 2008 (white) and 2009 (yellow) outbreak locations. The red arrow shows the location of the first outbreak in the Basque Country (Oiartzun, Gipuzkoa) (adapted from García-Lastra et al. 2012).

10. TAXONOMY, DISTRIBUTION AND PHENOLOGY OF THE MAIN BTV VECTORS IN SPAIN AND PORTUGAL

10.1. Taxonomy and nomenclature

The taxonomic status of the most important *Culicoides* species groups in the Iberian Peninsula (Imicola, Obsoletus and Pulicaris) involucrated as BTV vectors has remained confused over the last decade, with certain systematic imbroglios. The main species candidates as BTV vectors are *Culicoides imicola*, *Culicoides obsoletus* group (*C. obsoletus*, *C. scoticus*), *C. chiopterus*, *C. dewulfi* and *Culicoides pulicaris* complex (*C. pulicaris* and *C. lupicaris*). The correct nomenclature to refer to these species is briefly explained below.

Moreover, taxonomy is handicapped due to the small size of biting midges (requiring dissection and mounting techniques) and the lack of taxonomists in Europe as well as numerous unresolved synonymies and morphological variants. Indeed, the taxonomy of some *Culicoides* males, which are usually especially useful in the taxonomy of this family, are still unknown in several species due to their lower captures levels than females.

The improvement of molecular technology and rigorous studies of morphological taxonomy in the last years have revealed new insights to these species (Garros et al. 2010; Pili et al. 2010).

i) Subgenus *Avaritia*

- **Imicola group** includes seven Afrotropical species with *C. imicola* as the single species inhabiting the Iberian Peninsula.

- **Obsoletus group** includes six species in Holarctic region, of them *C. obsoletus* and *C. scoticus* supported their status as true species and must be included and named within the **Obsoletus complex** together with *C. montanus*, a rare species also present in the Iberian Peninsula.

- **Chiopterus group** includes *C. chiopterus* and *C. dobyi*, with *C. chiopterus* species distributed in the Iberian Peninsula. This species was previously included within the Obsoletus group.

- **Dewulfi group** includes *C. dewulfi*, a species which should not be considered part of the Obsoletus group because is phylogenetically closer to *C. imicola* (Schwenkenbecher et al. 2009).

ii) Subgenus *Culicoides*

- **Pulicaris group**. The specific number of species within the subgenus *Culicoides* in Palearctic region is unknown. In the Iberian Peninsula, 10 species are described. Pagés et al. (2009) sorted two main groups with specimens collected from Catalonia (Spain): Firstly, the **Pulicaris complex** comprised two sibling species: *C. lupicaris* and *C. pulicaris*, the last one with two forms.

- **Newsteadi group** with at least four species included within this group: *C. newsteadi* (various forms), *C. punctatus, C. impunctatus* and *grisescens.*
  Secondly, the **Fagineus complex** with *C. fagineus, C. subfagineus* and a third non-described species. *Culicoides flavipulicaris* as a single species closely related to the previous ones.

Nevertheless, some species were part of a complex with other cryptic species. Nolan et al. (2007) also studied in United Kingdom some species from this group, but no cryptic species were reported in the subgenera *Culicoides* with subsequently less complexity and more clear results than Pagés et al. (2009).

10.2. Distribution and phenology

The presence of the *Culicoides* vectors species is well-represented in the whole Peninsula. However, important differences are found when comparing the north region to the southern Spanish region.

In the north of Spain, *C. obsoletus* is markedly more abundant than *C. imicola*, in contrast to southern Spain where *C. imicola* is more common (Calvete et al. 2009). Perez et al. (2012) reported the *Culicoides* species composition in the whole Andalusia region, showing that in all the provinces sampled *C. imicola* was in higher numbers than *C. obsoletus* group (6 times), and *C. pulicaris* group was also slightly common than *C. obsoletus* group. In general, some species such as *C. chiopterus, C. dewulfi*, and *C. scoticus* are less common in comparison to *C. obsoletus s.s.* and *C. pulicaris* in comparison to *C. lupicaris* s.s. In the present thesis, the percentage distribution of these vectors in the Basque Country was: *C. obsoletus* group over 75%, followed by *C. pulicaris* group (10%) and only ten specimens of *C. imicola* (nine in Gipuzkoa and one in Biscay) (González et al. 2013a). In mainland Portugal, *C. imicola* accounted for 75.3% of the captures and the species belonging to the Obsoletus group 6.5% (69.6%, *C. obsoletus s.s.* and 30.4%, *C. scoticus s.s.*) (Ramilo et al. 2012).

With regard to the population dynamics, *C. imicola* is a multivoltine species and may produce several generations annually depending on temperature and other climatic variables (Braverman and Linley, 1988). Captures are seasonal and mostly occurred from May to November (summer/autumn). However, in Spain some differences are reported concerning population peaks. In general, *Culicoides imicola* populations peaked from August to October, with very low captures from December to April. For instance, in Andalusia, Ortega et al. (1997) captured more specimens in September and October than in August in 1990-1991, whereas Pérez et al. (2012) reported the presence of capture rates especially from May to October in 2007-2008, but concentrated mainly in August. In the Balearic Islands, the peak of adult populations of *C. imicola* occurs in October (Miranda et al. 2004), similarly in Extremadure, with high number of this species from September to October.

Culicoides obsoletus also displays several generations depending on previously mentioned factors. In contrast, in Spain *C. obsoletus* population peaks occur earlier, between May and July in general, with scarce collections from November to March. An extensive discussion is provided in **Chapter 2.**

11. FACTORS DETERMINING THE SPREAD OF BTV IN EUROPE

The unprecedented rapid expansion of BTV in Europe has been linked to various discussed factors, with different plausible explanations (Tabachnick, 2010):

i) **Climate change:** Purse et al. (2006) suggested that the BTV spread was driven by recent changes in European climate that allowed increased virus persistence during winter, the northward expansion of *C. imicola*, and beyond this vector's range, the transmission by indigenous European *Culicoides* such as *C. dewulfi*, *C. chiopterus*, *C. pulicaris* and *C. obsoletus* s.l. Subsequently, this species have showed to be efficient vectors involved in BTV transmission. This hypothesis is reinforced by temperature data in Europe. Seven of the warmest years from 1958 to 2007 occurred from 1998 to 2006, and 2006-2007 was the warmest on record (Wilson and Mellor, 2008). However, other environmental factors should not be dismissed as possible contributing factors (Gould and Higgs, 2009).

ii) **Wind transport:** many investigations have focused on the implications of wind as a long-range dispersal agent of *Culicoides* associated with suitable winds at certain velocity and air temperature. For this reason, wind is considered one of the most important and unpredictable ways of incursions of *Culicoides*-borne pathogens (i.e. BTV) onto islands and across large water bodies in the absence of animal movement. Nevertheless, wind-climatic models are useful tools for the prediction of *Culicoides* incursion routes, retrospective analyses of outbreaks and risk of the introduction of infected *Culicoides* species into different free vector areas of Europe (Hendrickx et al. 2008). In Spain, three serotypes (BTV-1, 2 and 4) may have entered the country in *Culicoides* transported by wind (Pérez de Diego et al. 20013). Infected *Culicoides* could have been transported by wind from Sardinia to the Balearic Islands, thereby causing the recorded bluetongue outbreaks (Alba et al. 2004). Indeed, meteorological data about wind (speed, altitude and trajectory) were used to explain the bluetongue virus (BTV-1) outbreak in the Basque Country in 2007–2008. It was proposed that the most likely scenario was the arrival of BTV infected *Culicoide*s midges from warm air masses from the south of the Iberian Peninsula (García-Lastra et al. 2012). Similarly, Martínez-López et al. (2009) estimated the potential introduction of *Culicoides* using Dust Regional Atmospheric Model (DREAM) from North

Africa to Spain. These results revealed that *Culicoides* introduction might occur through the South-Eastern Iberian Peninsula and Canary Islands, and sporadically from the Balearic Islands. The months from April to July were found to be at highest risk for *Culicoides* introduction.

iii) **Import of exotic animals:** in some parts of Europe, an increase in the number of animal parks added to an increase in exotic wildlife imported from BTV-endemic regions could be related to the increase of the likelihood of BTV entrance into free areas, its spread and epidemiology. One of the problems is that the majority of imported animals were not tested for BTV and thus they would have acted as reservoirs for the disease and even carry native infected *Culicoides* midges upon their bodies. In the case of Spain, animal theme parks increased by 230% between 1965-1995 (Tabachnick, 2010).

iv) **Import of domestic livestock:** the import of infected domestic livestock (sheep, goats, cattle) is reported as an important channel for disease introduction from BTV infected areas to free-disease sites (Wilson and Mellor, 2010) as BTV-8 may have entered Spain (Pérez de Diego et al. 2013) One of the most common problems is the lack of control in certain regions, transport of unvaccinated animals and also illegal commerce, especially in the first outbreak episodes.

v) **Changes in wildlife animal populations:** the high density of wild red deer, which has increased substantially in Western Europe during the last 20 years, has raised concerns about the potential role that unvaccinated European wild ungulates might play in maintaining or spreading the virus (Falconi et al. 2011).

vi) **Others:** changes in livestock management practises, changing practices associated with vaccinations, changing practices due to managing larger herds, changing animal practices due to higher temperatures and changes in the habitats of *Culicoides* could all contribute or cause changes in BTV epidemiology. A considerable number of alternative explanations for the new BTV episystem in Europe in addition to climate-driven hypotheses, and few of the alternative hypotheses have been adequately explored (Tabachnick, 2010).

12. STRATEGIES FOR *CULICOIDES* CONTROL

Control of biting midges requires an holistic knowledge of different eco-biological aspects of the *Culicoides* target species. The control of *Culicoides* has been previously neglected until they were known to be disease transmitting, except in those places where the enormous populations of *Culicoides* were responsible for annoying episodes of harmful bites, and the strategy of vaccination was used as the first line of defence. In fact, it is rarely possible to eliminate vectors completely from an area, so vector control should be judged only as a risk mitigation measure and should therefore always be used in concert with other control strategies (EFSA, 2007).

Most attempts to control *Culicoides* were carried out in the past decades; especially by adulticide residues on foliage, walls, and screens without effective results (Kettle, 1949, 1962). Other different control measures were implemented in the mid-twenties: aerial sprays, fogging, proofing of houses with wire gauzes, residual insecticides, smoke spots and pyrethrum coils and different ranges of repellents among others (Reye, 1964).

The interpretation of reported control measure results are often hindered by the participation of a great range of factors that interfere in their biology. In general, published works only consider one or two of the 11 possible control measures that we proposed (Fig. 19; Table 2). For instance, a combination of various techniques as an appraisal to protect livestock against *Culicoides* midges was carried out by Burkhard et al. (2009). In that work, the use of insecticide-treated mosquito fences and direct insecticide pour on treatment (deltamethrin followed by chlorpyrifos/cypermtehrin treatment) was applied at different dates. Curiously, no successful reduction in the feeding habits and number of specimens was obtained by these authors.

Fig. 19. An example of control by nematodes. *Culicoides* spp. naturally parasitized by nematodes.

A current control trend technique is based on push and pull strategies, which are defined as: " the behavioural manipulation of insect pests and their natural enemies via integration of stimuli that act to make the protected resource unattractive or unsuitable to the pests (push) while luring them toward an attractive source (pull) from where the pests are subsequently removed " (Cook et al. 2007) (Fig. 20).

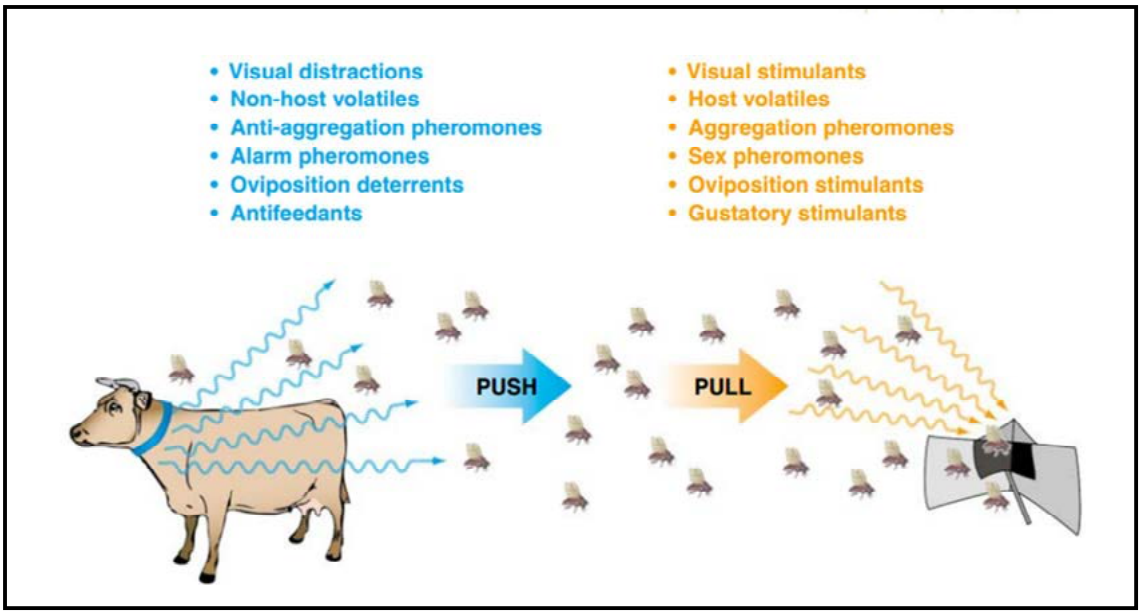


Fig. 20. The push-pull strategy: diagrammatic representation of the components and generalized mode of action (Cook et al. 2007).

These strategies have been used in different spheres such as subsistence farming, intensive arable agriculture, forestry, horticulture and urban pests. However, in the control of mosquitoes and midges there is poor research and lack of experimentation (Cook et al. 2007).

Appropriate chemicals could be used to prevent host location by "pushing" biting insects away from human and animal hosts via inhibitory or repellent mechanisms and at the same time, species-specific chemicals with attraction effect or newly identified attractants can be used as baits in traps to 'pull' the insects into traps and away from potential hosts (Logan and Birkett, 2007).

In the case of "push strategies," successful studies with repellents (synthetic, natural repellents and non-host volatiles) have been performed in laboratory and field experimentation. Similarly, host-derived semio-chemicals are being investigating from natural components released from "non-attractive or unattractive host" as new potential repellents against biting midges. In addition, further research is necessary in the study of

antifeedants, oviposition deterrents and antiaggregation pheromones in *Culicoides* biting midges.

Similarly, “pull strategies” are currently insufficiently studied or developed for the capture of a significant quantity of individuals, or in the reduction of the population of *Culicoides* in an area, with the exception of certain chemicals such as carbon dioxide, 1-octen-3-ol, or blends of these, which usually show promising results in some *Culicoides* species (Logan and Birkett, 2007).

However, one of the most useful tools as pull components in Integrated Pest Management (IPM) is the use of sex and aggregation pheromones. As indicated in **Section 6.3,** only pheromone evidences have been found in some *Culicoides* species and for this reason, pull components based on pheromone cues are currently ineffective in most of the *Culicoides* species. Moreover, these pheromones would possibly attract only males without direct veterinary relevance.

Sophisticated combinations of visual stimulants, with selective lures equipped with powerful traps (optimum electromagnetic spectrum) could be exploited in future IPM. The role of natural odours derived from breeding sites, as oviposition sites, is also an interesting research area that remains largely unexplored.

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Table 2. Summary of the main actions to control *Culicoides* (adults and larvae) biting midges and vaccines.

	Method to control	Target	Actions	Products used/ Methods	Problems	Efficacy
1	Insecticide-adulticide (large scale)	Adults	Fumigation: with vehicle-mounted fogging machines, light aircrafts, fumigant applicators or manually	- Organophosphates (malathion/naled) - Pyrethroides (Cypermethrin, deltamethrin, diazinon, permethrin, resmethrin, cyhalothrin)	Factors implicated in their effectiveness: volume, chemical quantities, kind of active compound, method, season and time of the day, species goal, kind and thickness vegetation and others. High residual effect over other related species, animals and plants (mainly in aquatic organisms)	Short-term: Medium-high Long term: Low
2	Insecticide-larvicide (micro scale)	Larvae		- Formerly: DDT, pyrethroides, pyrethrins - Now: Organophosphates (temefos, fention, chlorpyrifos) and ivermectin		Low
3	Micro-habitat removal (large scale)	Larvae	Mechanical removal of susceptible breeding sites and/or modifications of the optimal larval conditions to other incompatible with their survival (composting machinery)/larvicides	- Mechanical actions: Heavy machinery/ drainage actions - Larvicides (Organochlorines and Organophosphates): DDT, temefos, chlorpyrifos, pyrethrins, fention, dieldrin, chlordane, BHC	Depend on several factors: Capital outlay, recurring cost, accessibility, extent are	High if the work is well done
4	Micro-habitat removal (micro scale)	Larvae	Removal of animal litter, avoid drinking trough overflowing, dry or cover pool water with sand, take manure away from farm setting or dry or cover it with canvas, avoid run off areas by means of tube systems to tank/larvicides	- Few and cost-effective measures: enough to reduce the breeding sites of some *Culicoides* species - Larvicides	Actions only valid when *Culicoides* breeding sites are specific and no scattered	Medium-high (no publications are available)
5	Livestock direct-application	Adults	Topical application: Insecticide-impregnated animal skin with insecticide (poor on application/inmersion/crotal), with pipetes or with manually spraying. Systemic application: intracutaneous/ subcutaneous	- The most common: Pyrethroids (deltamethrin/ permethrin) - Others: Cypermethrin, flumethrin, fenvalerate - Organophosphates: Diazion, chlorpyrifos -Other common compounds: Pyperonil butoxide - Inyectable: ivermectin	Calculate the optimal lethal doses, achieving optimal dispersal of the insecticide over the whole skin (specially in susceptible areas where *Culicoides* bites) and physiological characteristics of the animal (e.g. hair density)	Medium-high (if problems previously mentioned are solvent)

	Method to control	Target	Actions	Products used/Methods	Problems	Efficacy
6	Mesh-netting protection/stabling livestock	Adults	Use of mosquito fences and/or screens to provide physical barriers that prevent attacks by biting midges inside the livestock sheds	- The best is the use of insecticide-treated mosquito fences; now are commercially available chemical nets with deltamethrin treatment - Meshes can be combined with insecticides and also with repellents compounds (very different interpretations and results have been reported)	By themselves are impractical. Mesh size is important (< 0,9 mm^2 prevent most *Culicoides* access, but extra problems in animal health could entail. Optimal width: 1 x 2 mm. Material of the net is also important (better results with polyester fences). It is almost impossible to cover all the entries in the livestock sheds. Evaluate the exophilic/endophilic behaviour of the *Culicoides* present in treated-area	-By themselves: low -Combined with insecticide: different interpretations, usually reduction in the number of *Culicoides* individuals are recorded
7	Repellents	Adults	The use of semio-chemical compounds with repellence activity against targeted-*Culicoides* specimens	- Chemical compounds and plant derived compounds - Wide range of products commercially available and others in research, mainly natural botanical products	Skin alterations, efficacy and durability, volatility, odour, range of insects, meteorological resistant… Difficulties of experimentation over animals and humans	-Synthetic products: Long-term effectiveness but less safety - Botanical compounds: Short-term but safety
8	Attractants	Adults	The use of semio-chemical compounds with attractive properties against targeted-*Culicoides* specimens	- Host-derived compounds and chemical products (carbon dioxide, octenol, acids, acetone, phenols and blends of them)	Each species reacts in a different way. In general, attractants are not as powerful as repellents. Their importance lies as push-pull strategies. Pheromones as attractive way are not considered very promising (except in few species of *Culicoides*)	Low by themselves and poor-studied
9	Massive capture	Adults	The use of control traps/devices to catch the major number of targeted-*Culicoides* specimens	- Suction traps (with powerful funs) equipped with attractive cues as light and /or semio-chemicals. Some companies purchase them as control measures but also are useful for monitoring studies	It is thought that a great quantity of traps is necessary to cover extensive areas. It is not cost-effective but a power supply is necessary and also requires adequate lures and light sources depending on different targeted-species	- Particular use (gardens): high when the midges population is low, if not, more traps are required. -Large scale: No long-term studies are available, but short-term seems to be not very promising
10	Biorational pesticides	Adults and larvae	The use of hormonal and biological control agents as way to fight against *Culicoides* specimens	- Hormones, predators and pathogens as bacteria, nematodes, fungi, virus...	Moderately studied in laboratory assays but poorly in field trials. Difficulties in results interpretations. Biological agents are curretly in development in applied entomology because are respectful with environment	In general low, except with some species of nematodes and some strains of fungal pathogens

	Method to control	Target	Actions	Products used/Methods	Problems	Efficacy
11	Vaccines	Host	Prophylactic immunization: vaccination programs of those susceptible host-animals of important diseases (BTV, AHS, SBV...)	- Formerly: Live attenuated-specific vaccine - Now: Inactivated-specific vaccines commercially available both monovalent or polyvalent vaccines	A great range of serotypes (8) have been circulating through Europe. There are some cases reported of bad-health animal conditions attributed to side effects of BTV vaccines (no agreement between specialist). Certain time is required to create specific vaccines depending on different immunology parameters	It is practical and effectiveness

Main consulted bibliography: Kettle, 1962; Linley and Davies, 1971; Day and Sjogren, 1994; Cilek and Kline, 2002; Mands et al. 2004; Satta et al. 2004; Cilek and Hallmon, 2005; EFSA, 2007; Carpenter et al. 2008; Mullen and Durden, 2009.

Note: Consult the Biocidal Products Directive (DIRECTIVE 98/8/EC). Annex V: product type 18 and 19 for more information about insecticides, repellents and attractants.

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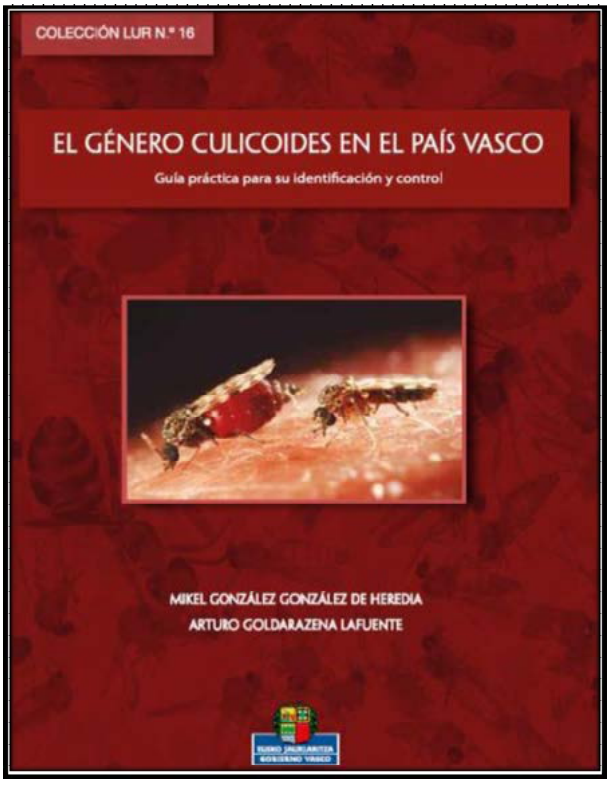
CHAPTER 1



Version of the chapter based on the following publication:

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Mikel González and Arturo Goldarazena

COLECCIÓN LUR N.º 16

The genus *Culicoides* in the Basque Country: Overview of *Culicoides* diversity

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THE GENUS *CULICOIDES* IN THE BASQUE COUNTRY: OVERVIEW OF *CULICOIDES* DIVERSITY

1. ABSTRACT

The book (Gonzalez and Goldarazena, 2011) contains a review of species of the genus *Culicoides* (Diptera: Ceratopogonidae) in the Basque Country. The work begins with an extensive introduction to the biology, economic importance, methods of study and control strategies of these dipterans and continues with chapters on the identification and the taxonomy of the group. A taxonomic studies of over 47 species along with their morphological descriptions, geographical distributions, as well as notes on their relevance as potential vectors of bluetongue virus are also reported. All taxonomic levels are included in a simple dichotomous key to separate the taxa described. The book contains 550 photographs of morphological details which enable the identification of each species and ends with an extensive bibliography of scientific works which have been cited throughout the text.

This work is undoubtedly an important tool for those who are interested in the study of these insects, both from the applied point of view (farmers, veterinarians, applied entomologists) and for the study of biodiversity. It will also serve as a valuable tool to confront the problems that come cyclically due to the arrival of invasive species that could be vectors of virus and to the threat that the global climatic change leads to species distribution.

2. KEY WORDS Biodiversity, Basque Country, *Culicoides*, Illustrated key, Wings

3. INTRODUCTION

From this book edited in Spanish that includes the work carried out from 2007 to 2009, we have selected and updated the most relevant results about the diversity of *Culicoides* species in the Basque Country from 2007 to 2013. It comprises all the species captured in that region through different entomological monitoring programs as well as other types of trappings. The number of captured species have increased from 47 (González and Goldarazena, 2011) to 52 identified species to date.

Shown in the current chapter are the wing patterns of all female species, five plates with descriptions about the new species discovered posterior to the publication of the book and also an updated dichotomous key for the identification of 52 *Culicoides* midges (females and males) found in the Basque Country region. The key was illustrated with pictures of the main characters to facilitate the understanding of the key diagnosis. Finally, we include some biodiversity data of other ceratopogonids in our region.

The aim of this chapter is to report the biodiversity of *Culicoides* and develop an identification key for the species from Basque Country.

4. MATERIAL AND METHODS

All the material captured and identified for all the species of *Culicoides* was gathered from fresh material collected during several monitoring programs mainly carried out with CDC (Center Disease Control)-Blacklight (UV) trap-model 1212 (John W. Hock company, Gainesville, Florida) in a total of 11 sheep farms from the Basque Country and seven natural habitats from the province of Alava. Voucher specimens were stored in slide-mounted preparations as well as in alcohol glasses in the Entomology Laboratory of Neiker-Tecnalia (Basque Institute of Agricultural Research and Development, Vitoria, Spain).

All the photographs included in this volume have been taken by M. González. Slide-mounted specimens were photographed with a Leica DM 4500B microscope coupled with a Leica camera DF C300FX with the technique interdiferencial contrast (DIC). Helicon focus software was used for processing images, overlaying all the images in a new one. Imperfections of pictures were restored and cleaned up with Photoshop 13.0 program.

The methodology is described in detail in González and Goldarazena (2011), (pp. 69-72; Fig. 1) and a brief summary is also provided in González et al. 2013a (**Chapter 2**).

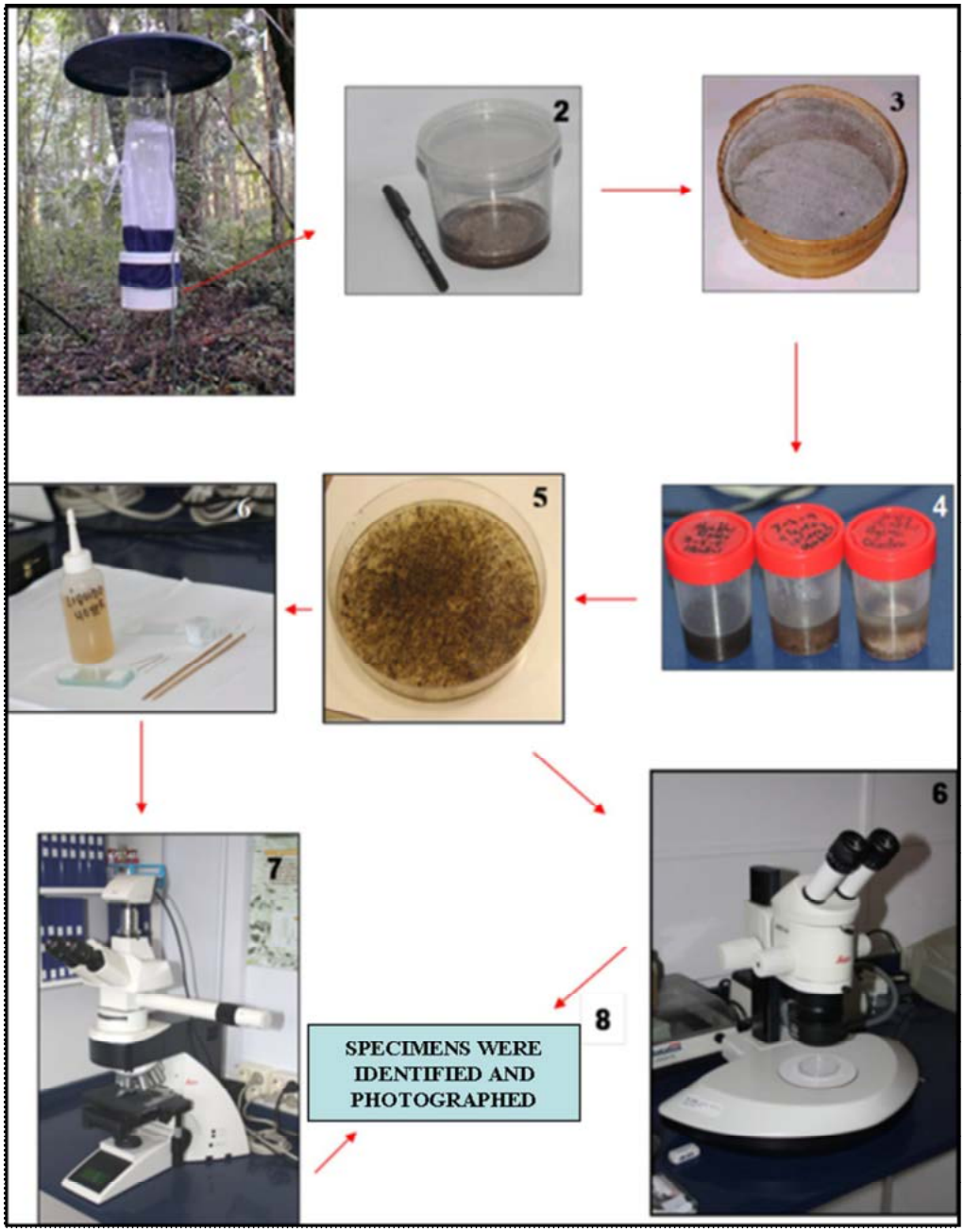


Fig. 1. Schedule of activities implemented in the processing of *Culicoides* **(1)** CDC-traps **(2)** Jar with collected insects **(3)** Sieve **(4)** Stored and labeled bottles **(5)** Fresh specimens in alcohol **(6)** Material for slide-mounted preparations or direct examination under stereomicroscope **(7)** Microscope with a coupled camera **(8)** Identification and photographs.

5. RESULTS

5.1. List of *Culicoides* species captured in the samplings

Subgenus *Avaritia* Fox, 1955……..……...

***C. chiopterus* (Meigen, 1830)**
***C. dewulfi* Goetghebuer, 1936**
***C. imicola* Kieffer, 1913**
***C. obsoletus* (Meigen, 1818)**
***C. scoticus* Downes and Kettle, 1952**

Subgenus *Beltranmyia* Vargas, 1953…….....

***C. circumscriptus* Kieffer, 1918**

Subgenus *Culicoides* Latreille, 1809…….……....

***C. fagineus* Edwards, 1939**
***C. flavipulicaris* Dzhafarov, 1964**
***C. impunctatus* Goetghebuer, 1920**
***C. lupicaris* Downes and Kettle, 1952**
***C. newsteadi* Austen, 1921**
***C. pulicaris* (Linnaeus, 1758)**
***C. punctatus* (Meigen, 1804)**
***C. subfagineus* Delécolle and Ortega, 1998 ***

Subgenus *Monoculicoides* Khalaf, 1954……..…….

***C. nubeculosus* (Meigen, 1830)**
***C. parroti* Kieffer, 1922**
***C. riethi* Kieffer, 1914**
***C. stigma* (Meigen, 1818)**

Subgenus *Oecacta* Poey, 1853…….……..

***C. alazanicus* Dzhafarov, 1961**
***C. brunnicans* Edwards, 1939**
***C. cataneii* Clastrier, 1957**
***C. clastrieri* Callot, Kremer and Deduit, 1962**
***C. comosioculatus* Tokunaga, 1956**
***C. duddingstoni* Kettle and Lawson, 1955 ***
***C. festivipennis* Kieffer, 1914**
***C. furcillatus* Callot, Kremer and Paradis, 1962**
***C. gejgelensis* Dzhafarov, 1964**
***C. griseidorsum* Kieffer, 1818**
***C. heliophilus* Edwards, 1921**
***C. kibunensis* Tokunaga, 1937**
***C. kurensis* cf. Dzharafov, 1960**
***C. maritimus* Kieffer, 1924 ***
***C. longipennis* Khalaf, 1957**
***C. odiatus* Austen, 1921 [a]**

***C. paradisionensis* Boorman, 1988**
***C. pictipennis* (Staeger, 1839)**
***C. poperinghensis* Goetghebuer, 1953**
***C. pseudoheliophilus* Callot and Kremer, 1961 ***
***C. santonicus* Callot, Kremer, Rault and Bach, 1966**
***C. semimaculatus* Clastrier, 1958**
***C. shaklawensis* Khalaf, 1957**
***C. simulator* Edwards, 1939**
***C. truncorum* Edwards, 1939 ***
***C. univitattus* Vimmer, 1932**
***C. vexans* Staeger, 1839**

Subgenus *Silvaticulicoides* Glukhova, 1972…….…….…….……..

***C. achrayi* Kettle and Lawson, 1955**
***C. fascipennis* (Staeger, 1839)**
***C. pallidicornis* Kieffer, 1919**
***C. picturatus* Kremer and Deduit, 1961**
***C. subfasciipennis* Kieffer, 1919**

Subgenus *Wirthomyia* Vargas, 1973…….…….…….……..

***C. minutissimus* (Zetterstedt, 1855)**

Subgenus *Pontoculicoides* Remm, 1968….……...……

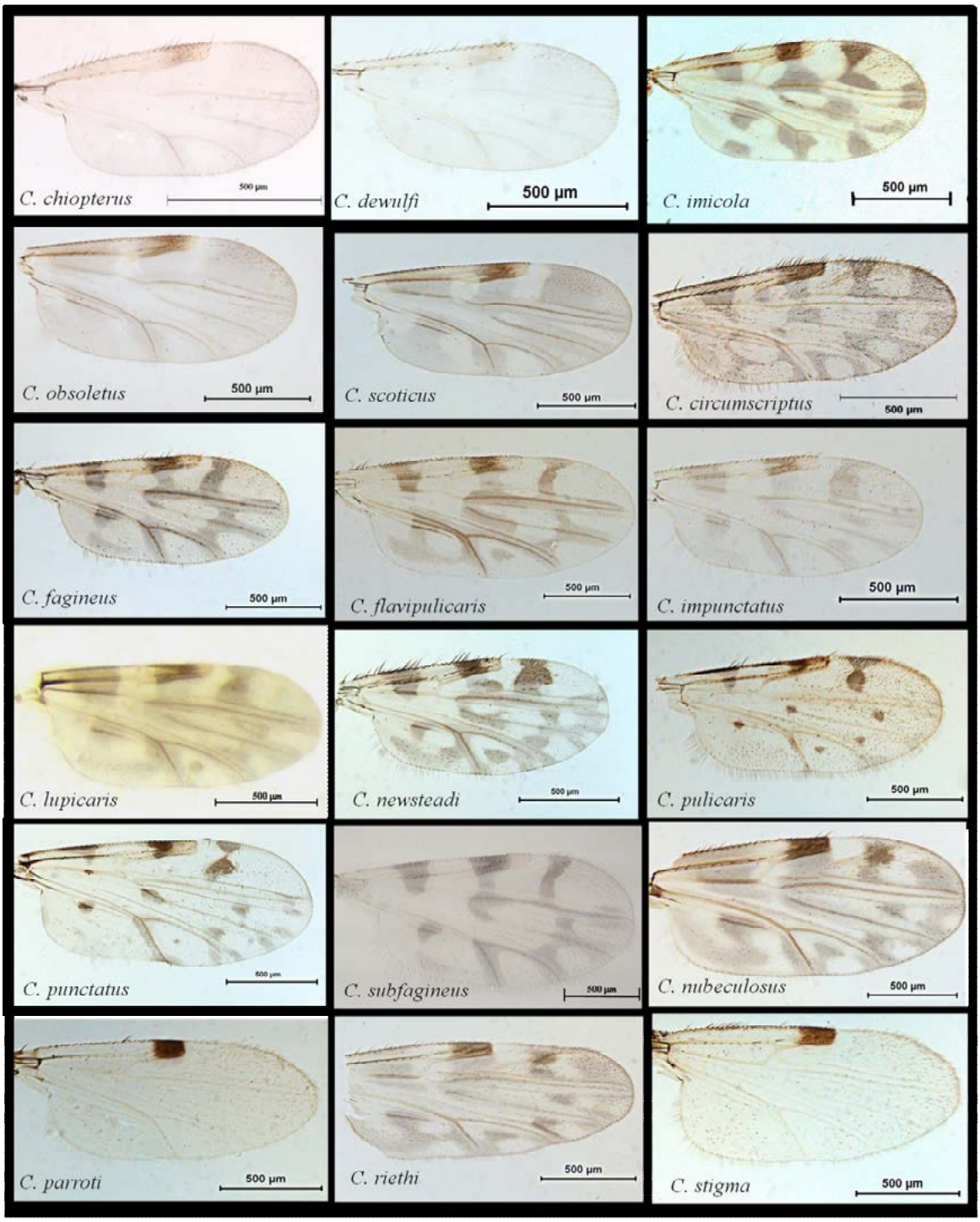
***C. tauricus* Gutsevich, 1959**

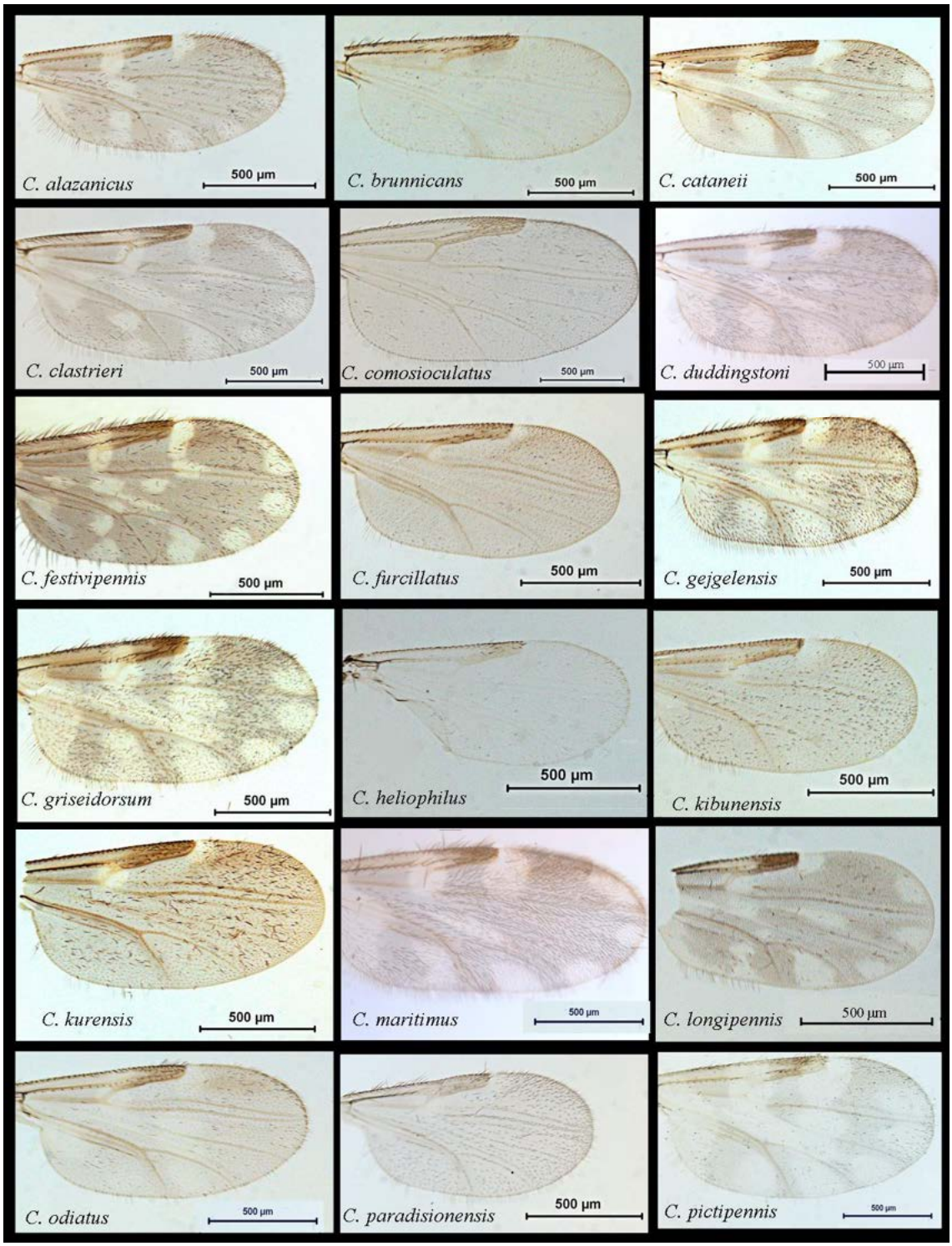
……..

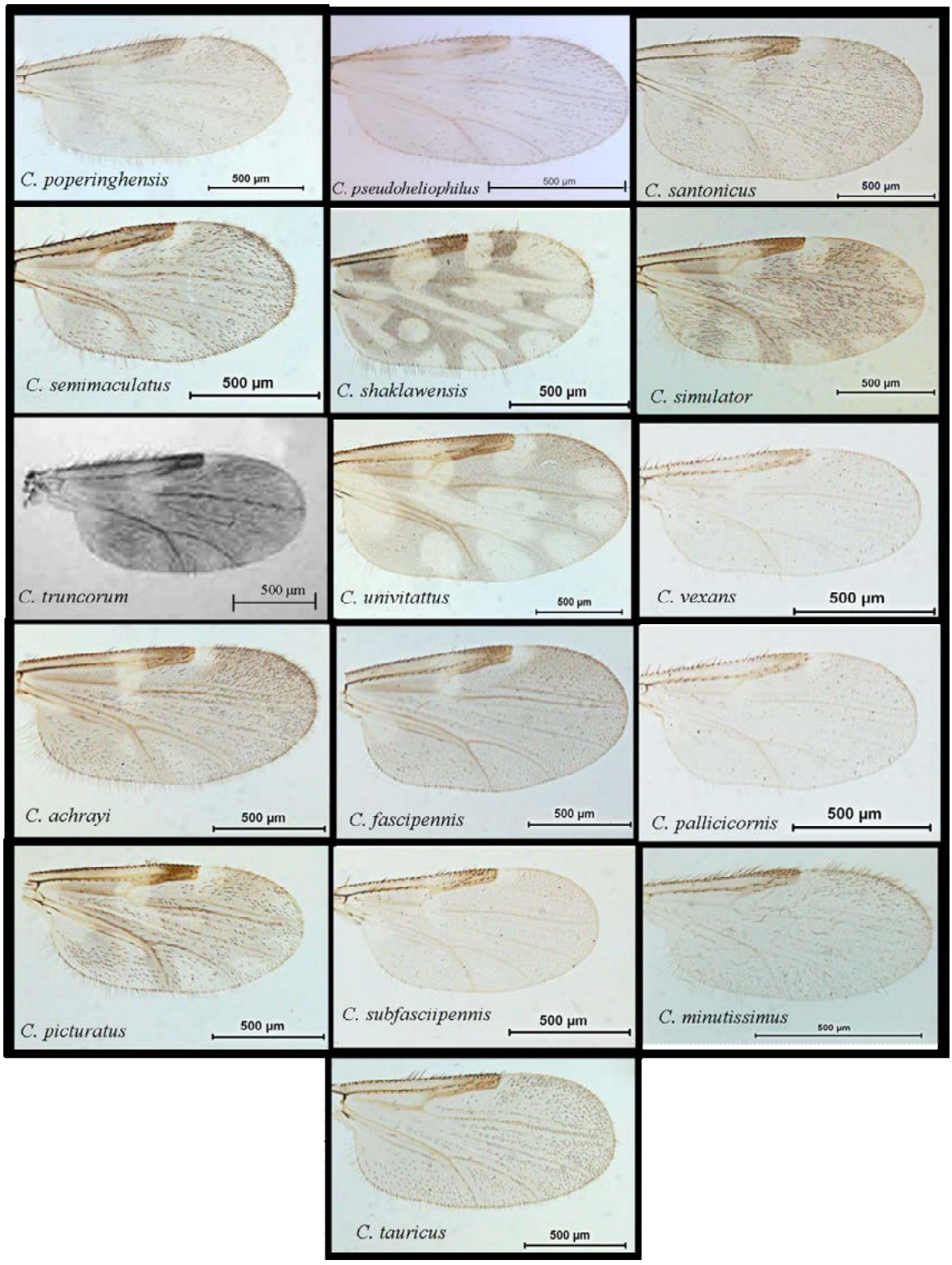
***** Asterisk denotes species not mentioned in the book, that were found later in field samplings (descriptions and plates are given in page 121-129)

[a] *C. odiatus* could be also placed on an unclear subgenus.

5.2. Wing pattern of the 52 *Culicoides* species trapped in the Basque Country
(*apparition order in correspondence with the previous list*)




C. alazanicus
500 µm
C. brunnicans
500 µm
C. cataneii
500 µm
C. clastrieri
500 µm
C. comosioculatus
500 µm
C. duddingstoni
500 µm
C. festivipennis
500 µm
C. furcillatus
500 µm
C. gejgelensis
500 µm
C. griseidorsum
500 µm
C. heliophilus
500 µm
C. kibunensis
500 µm
C. kurensis
500 µm
C. maritimus
500 µm
C. longipennis
500 µm
C. odiatus
500 µm
C. paradisionensis
500 µm
C. pictipennis
500 µm


C. poperinghensis
500 µm
C. pseudoheliophilus
500 µm
C. santonicus
500 µm
C. semimaculatus
500 µm
C. shaklawensis
500 µm
C. simulator
500 µm
C. truncorum
500 µm
C. univitattus
500 µm
C. vexans
500 µm
C. achrayi
500 µm
C. fascipennis
500 µm
C. pallicornis
500 µm
C. picturatus
500 µm
C. subfasciipennis
500 µm
C. minutissimus
500 µm
C. tauricus
500 µm

5.3. Plates of the species non-described in the book: González and Goldarazena (2011)

C. duddinsgtoni ……..(Plate 1)

Description:

♀ size 2 mm; Wing 1,26 mm.

Females: Eyes separated by less than one facet and no pubescent (Fig. 1). Antennae sensilla coeloconica on segments 3 to 15. Third maxillary palp swollen with a very deep sensory pit (Fig. 2). Brownish and pubescent wings with pale spots at the end of cells R_5, M_1, M_2, Cu and An, which are cut by the wing margins. A pale spot covering basal area of M_1 and other one along M to basal part of M_2. Pale spot on r-m and on area 2 but second radial cell dark. Basal cell with macrothichia (Fig. 3). Oval spermathecae without neck (Fig. 4).

Males: Ninth tergite with two well-develop apicolateral processes, quite long and with a imperceptible notch (Fig. 6). Ninth sternite with caudomedian excavation concave and its membrane speculate (Fig. 5). Apodemes (ventral roots) elongated and sharped. Body of aedeagus rectangular-shaped (Fig. 5). Parameres with a typical aspect and pointed tips.

Annotations: At first view, resembles *C. cataneii*/*C. gejgelensis* but also to *C. maritimus.* However, it could be separated from the mentioned species under stereomicroscope (high magnification) due to their very deep pit. Basal cell bears abundant macrotrichia as *C. maritimus.*

Biology: The type series was found breeding from mud from the edges of Scottish lochs. González et al. (2013a) found few specimens in mud and in standing water with roots of *Typha* spp. from a pond of Spain.

Distribution: From U.K. to Ukraine including most countries of north-central Europe and France. Absent in south Europe. Present in Sardinia and Corsica and also in near East (Georgia, Armenia and Azerbaijan) (Mathieu et al. 2010).

Feeding preferences: According to the molecular analyses of Lassen et al. (2012) and Pettersson et al. (2013) and its presence on bird-baited traps (Cerný et al. 2010), this species is considered as ornithophilic. The blood-meal of one specimen revealed having blood from an Eurasian jay (*Garrulus glandarus*).

Plate 1. *Culicoides duddingstoni* ♀ and ♂

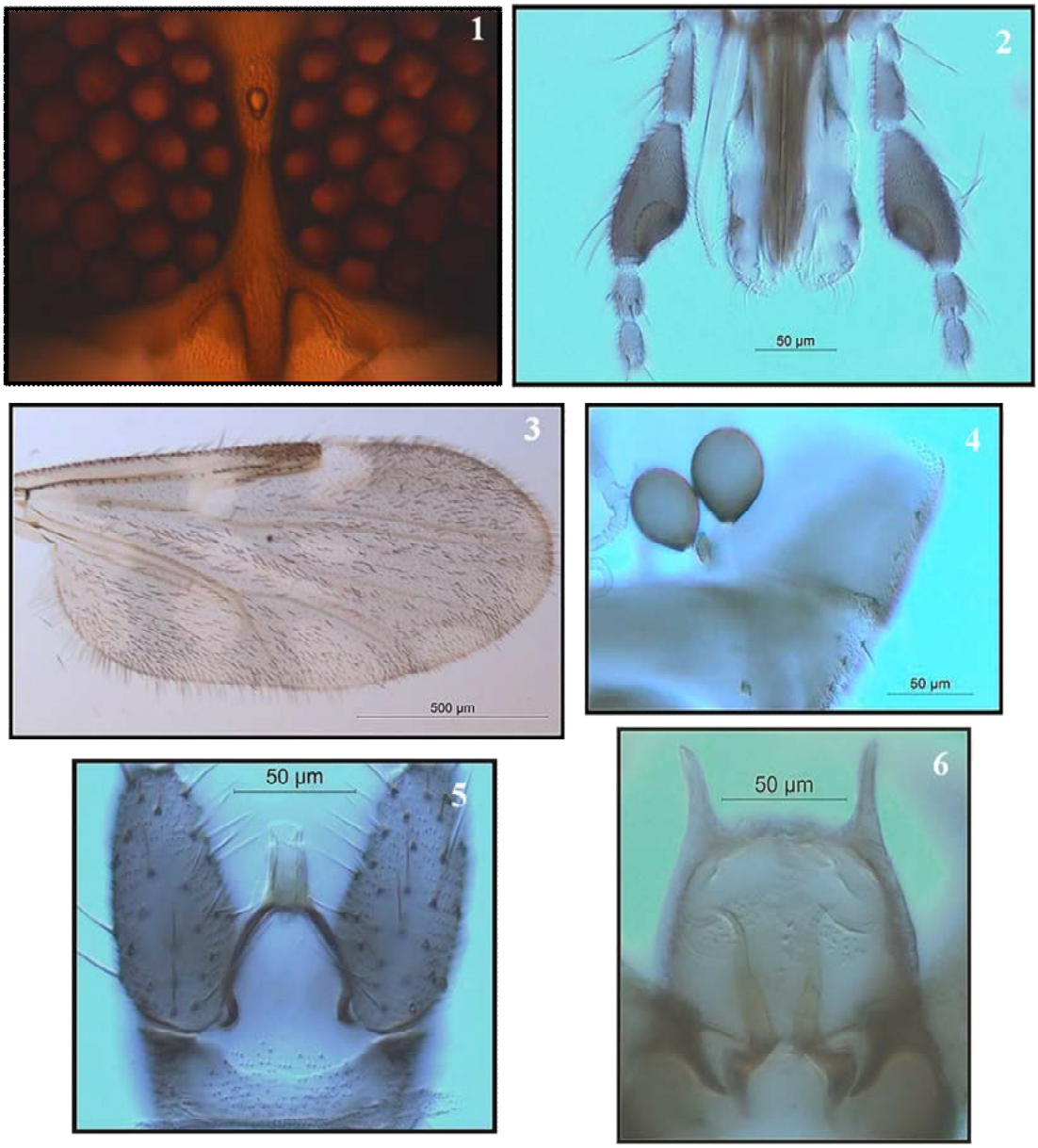


Plate 1: *C. duddingstoni*; (♀) Compound eyes (**Fig. 1**); Maxillary palpus (**Fig. 2**); Wing (**Fig. 3**); Spermathecae (**Fig. 4**); (♂) Ninth sternite and *aedeagus* (**Fig. 5**); Genitalia (**Fig. 6**).

C. maritimus …….. (Plate 2)

Description:

♀ size 2,1 mm; Wing 1,3 mm.

Females: Eyes completely separated by more than 1-1,5 facets and no pubescent (Fig. 1). Antennae sensilla coeloconica on segments 3 to 15. Third maxillary palp slightly swollen with a shallow sensory pit (Fig. 2). Brownish and pubescent wings with well-defined pale spots at the ends of cells R_5, M_1, M_2, (do not quite reach the wing margin) and Cu and An. A pale spot covering basal area of M_1 and other one on M joined to basal M_2 cell spot. Pale spot on area 2 but second radial cell dark. Pale spot over r-m joined to M spot. Basal cell with macrothichia (Fig. 3). Oval spermathecae without neck (Fig. 4).

Males: Ninth tergite with two well-develop apicolateral processes, quite long and divergent (Fig. 5). Margin of ninth tergite with a imperceptible notch. Apodemes (ventral roots) of medium size and sharpened. Ninth sternite with the caudomedian excavation concave and its membrane densely speculate (Fig. 6). Body of aedeagus triangular-shaped (Fig. 6). Parameres with a typical aspect and pointed tips.

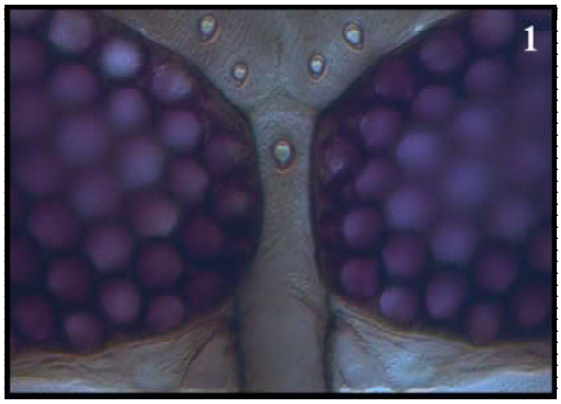
Annotations: At first view, resembles *C. pictipennis*. However, could be easily separated from it, according to the pale spot over area 2, which in that species cover part of the radial cell and in contrast to *C. maritimus* which is completely dark. Indeed, also resembles *C. duddingstoni*, but the separation of the eyes and the depth of the sensory pit among others serve to separate it. *Culicoides maritimus* pale spots are in general well-distinct and bigger than *C. duddingstoni*. There are also appreciable differences in the spots pattern.

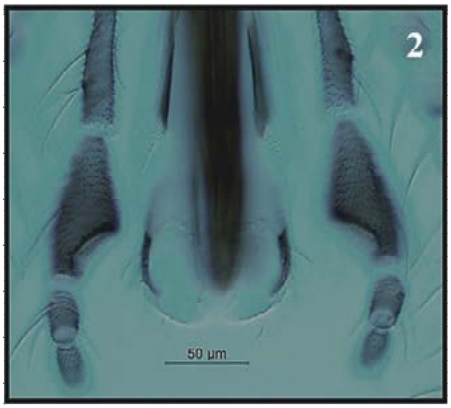
Biology: As its name suggests, it is found in coastal localities and particularly salt marshes (Culicoides.net, 2013).

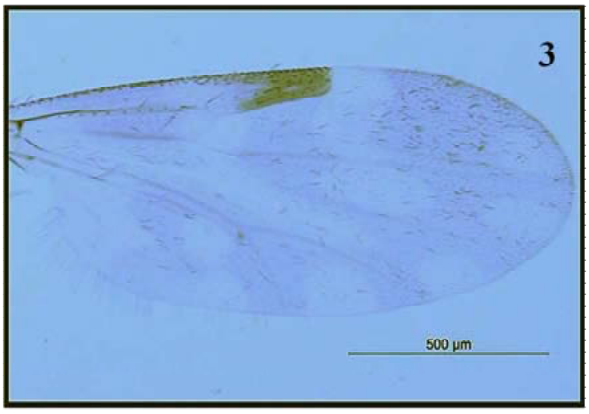
Distribution: Throughout Europe from U.K. to Russia, and countries bordering the Mediterranean. Absent in some European countries. North Africa and Near East (Mathieu et al. 2010).

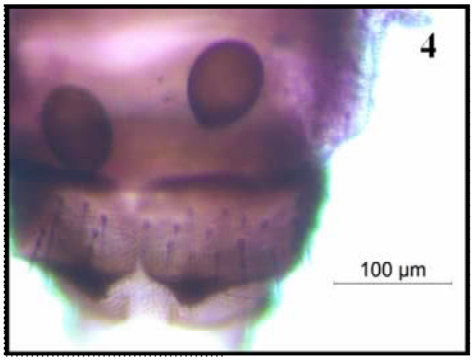
Feeding preferences: According to the studies carried out by Braverman et al. (2012) based on morphometric parameters of palps and antennal sensilla, this species matches with a bird feeder.

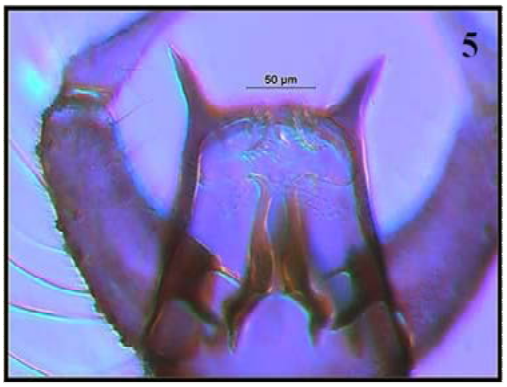
Plate 2. *Culicoides maritimus* ♀ and ♂











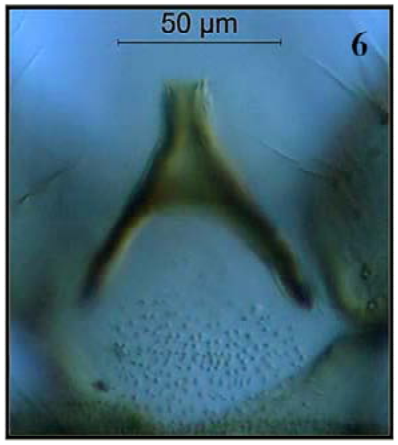


Plate 2: *C. maritimus*; (♀) Compound eyes (**Fig. 1**); Maxillary palpus (**Fig. 2**); Wing (**Fig. 3**); Spermathecae (**Fig. 4**); (♂) Genitalia (**Fig. 5**); Ninth sternite and *aedeagus* (**Fig. 6**).

C. pseudoheliophilus ……...……..... (Plate 3)

Description:

♀ small size specimens.

Females: Eyes well-separated by more than 1-1,5 facets and no pubescent (Fig. 1). Antennae sensilla coeloconica on segments 3 and from 13 to 15. Third maxillary palp slender, with a small single sensory pit (Fig. 2). Small wings with an indistinct and blurred pale spots pattern (Fig. 3). Spherical spermathecae with neck (Fig. 4).

Males: Ninth tergite with two well-develop apicolateral processes, quite thick. Absence of notorious notch in ninth tergite. Caudomedian excavation of ninth sternite concave and its membrane bare (Fig. 6). Apodemes (ventral roots) elongate, sharp and weekly curved (Fig. 5). Body of aedeagus trapezoidal-shaped (Fig. 6). Parameres short, stout and thick.

Annotations: At first view, the tones of the wing resemble *C. poperinghensis*, but it is a bigger species in size and spots and is also well-marked. Indeed, *C. pseudoheliophilus* is one of the few species with mouthparts without teeth in maxillae. *Culicoides pseudoheliophilus* wing pattern bears a very indistinct pale spots that usually are imperceptible and difficult to locate on the wing. This species is also reported as *C. albihalteratus.*

Biology: This species could be found in wild-habitats not associated with livestock farms (González et al. 2013b; Zimmer et al. 2013).

Distribution: Recorded in Spain, Portugal, France, Italy, Germany, Poland, Czech Republic, Belgium and Estonia (Mathieu et al. 2010).

Feeding preferences: Up to date no information is reported. The absence of teeth in maxillae do not means that this species could not suck on blood of vertebrates. i.e. *C. albicans* females species without teeth on both maxillae and mandibles have been reported as anthropophilic (Kremer, 1965).

Plate 3. *Culicoides pseudoheliophilus* ♀ and ♂

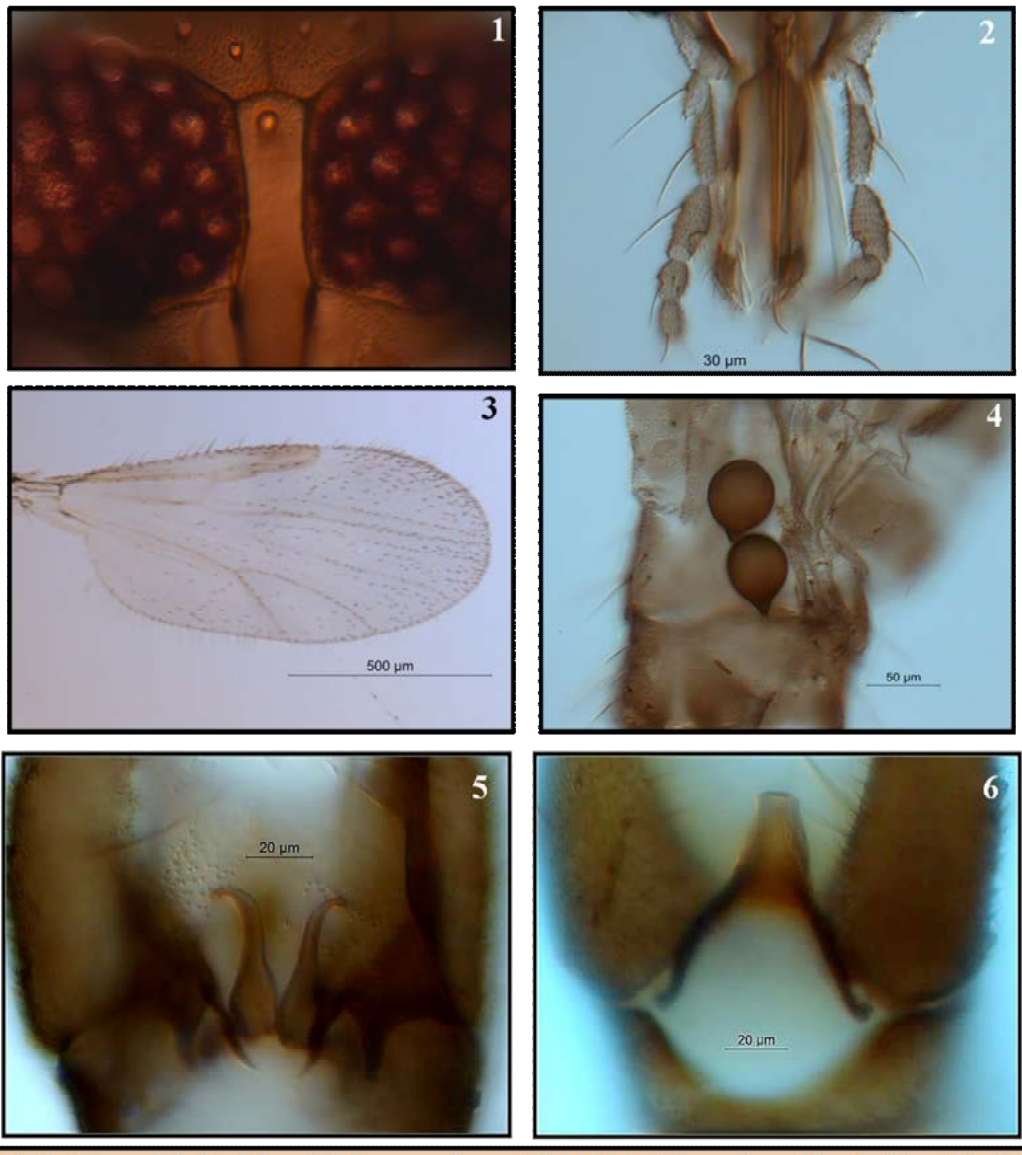


Plate 3: *C. pseudoheliophilus*; (♀) Compound eyes (**Fig. 1**); Maxillary palpus (**Fig. 2**); Wing (**Fig. 3**); Spermathecae (**Fig. 4**); (♂) Genitalia (**Fig. 5**); Ninth sternite and *aedeagus* (**Fig. 6**).

C. truncorum ……. (Plate 4)

Description:

♀ size 1,5 mm; Wing 1,15 mm.

Females: None captured in the present thesis. Eyes separated by 0,5-1 facets and no pubescent. Antennae sensilla coeloconica on segments 3, 5, 7, 9 and from 11 to 15. Third maxillary palp swollen with a deep sensory pit. The wing is pale, with vague pale spots, one over the r-m vein, one just beyond the second radial cell, and spots at the margin of the wing in R_5, M_1, M_2, Cu and An. Oval spermathecae without neck.

Males: Wing (Fig. 1). Ninth tergite with two short, sharp and straight apicolateral processes (Fig. 2). Absence of notch on posterior margin of ninth tergite. Ninth sternite with the caudomedian excavation concave and its membrane bare (Fig. 4). Apodemes foot-shaped (Fig. 3). Body of aedeagus small, elongated and pointed (Fig. 4). Tip of parameres fallen down.

Annotations: The spots pattern distribution could remember to *C. picturatus* and even *C. alazanicus.* However, even the tones of the wing are slightly different there are other several features to be easily separated.

Biology: This species probably breeds in tree holes, often in company with *C. fagineus* (Culicoides.net, 2013) and is also associated with wetland habitats (Zimmer et al. 2013).

Distribution: United Kingdom, Spain, Portugal, France, Belgium, Germany, Switzerland, Slovakia, Czech Republic, Denmark, Estonia, Ukraine and Russia, north Africa and near East (Mathieu et al. 2010).

Feeding preferences: Although no molecular data are available actually, there are enough evidences based on field trapping in nests that this species could be bird-feeder (Martinez-de la Puente et al. 2009; Votýpka et al. 2009; Cerný et al. 2010).

Plate 4. *Culicoides truncorum* ♂

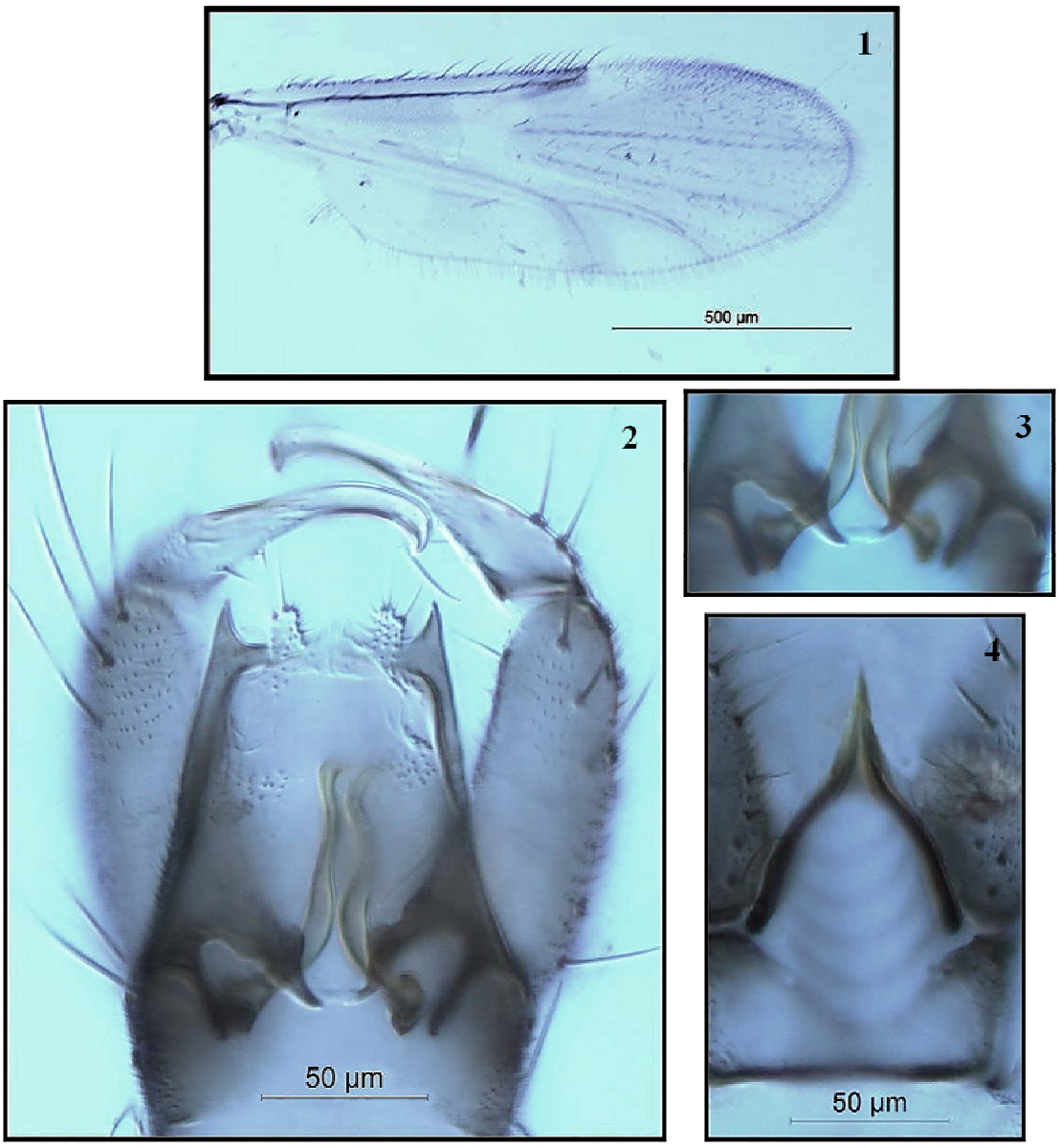


Plate 4: *C. truncorum*; (♂) Wing (**Fig. 1**); Genitalia (**Fig. 2**); Basistyle roots (**Fig. 3**); Ninth sternite and *aedeagus* (**Fig. 4**).

C. griseidorsum …… (Plate 5)

Description:

♀ Wing 1,31 mm.

Females: Females are described in González and Goldarazena (2011).

Males: Wing (Fig. 1). Ninth tergite with two well-developed apicolateral processes, quite long and divergent (Fig. 2). Absence of notorious notch in posterior margins of ninth tergite. Ninth sternite with a caudomedian excavation concave and densely spiculate. Apodemes (ventral roots) with medium size and sharpened (Fig. 2). Body aedeagus trapezoidal-shaped with the tip strongly convex (Fig. 3). Parameres stout with the typical aspect.

Annotations: Males figures seems to have not reported. These pictures are the only available pictures to date for this species.

Distribution: Spain, U.K, France, Belgium, Alemany, Poland, Italy, Greece and north of Africa (Mathieu et al. 2011).

Plate 5. *Culicoides griseidorsum* ♂

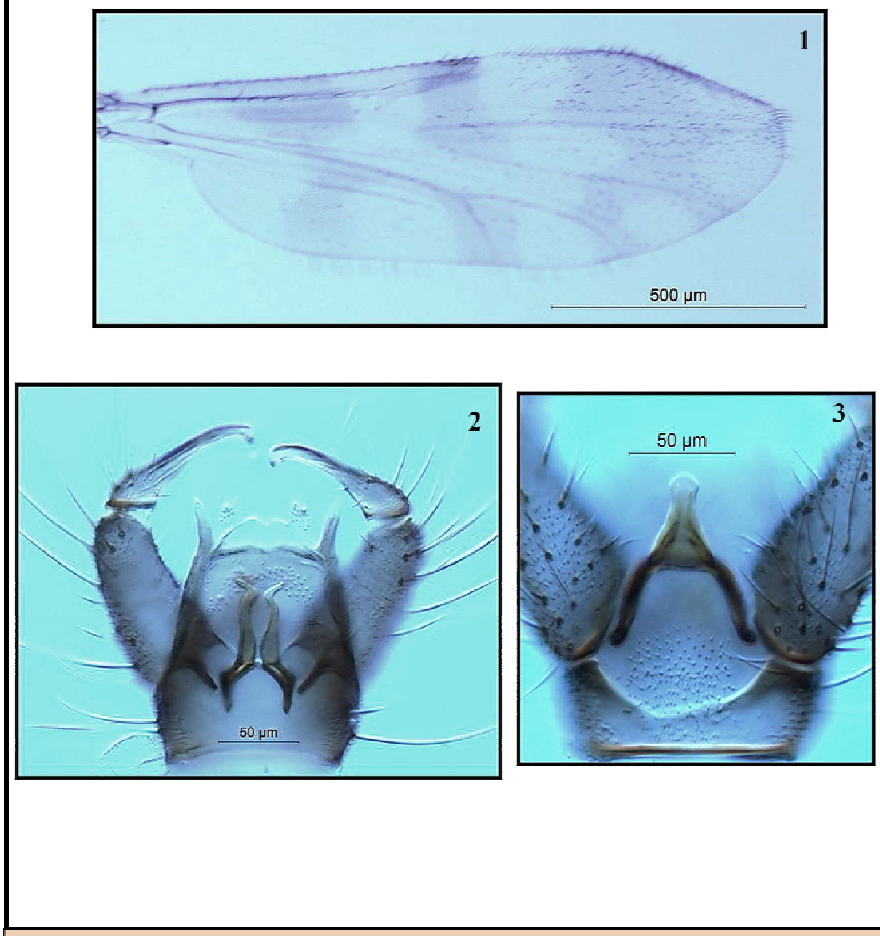


Plate 5: *C. griseidorsum*; (♂) Wing (**Fig. 1**); Genitalia (**Fig. 2**); Ninth sternite and *aedeagus* (**Fig. 3**)

5.4. Dichotomous key for *Culicoides* females identification (slide-mounted specimens). *Key based on Delécolle (1985) and Glukhova (2005)*

1. One spermatheca (**1**)……..……...2
Two or three functional spermathecae (**2, 3**)……...……..……...……6

2. Antennal sensilla coeloconica pattern on segments 3 to 14. Third segment of maxillary palp thick and with a conspicuous deep sensory pit (**4**). Presence of post-pharyngeal ornamentation (**5**)…….…….……..….***C. circumscriptus***
Antennal sensilla pattern on 3, 8 to 10. Third segment of maxillary palp thin and with a small-moderate sensory pit. Without ornamentation……...……..…..……...…3

3. Wing with single conspicuous dark spot on second radial cell, contrasting, light background (**6**)…….……..…….....4
Wing with evident spots pattern (**7**)……..……..……..……...5

4. Spermathecae kidney-shaped with a wide neck (**8**). Halters greyish (**9**)…..…..***C. parroti***
Spermathecae spherical with finger-shaped processes and narrow neck (**10**). Halters palish (**11**)……...…***C. stigma***

5. Thorax with a pale spot on *scutellum* (specimens in alcohol). Spermatheca retort-shape with wide neck (**12**)……..……***C. nubeculosus***
Thorax with an entirely dark *scutellum* (specimens in alcohol). Spermatheca oval with narrow neck (**13**)……..……..……***C. riethi***

6. Three functional kidney-shaped spermathecae (**14**)…..……..…..…....….***C. tauricus***
Two functional spermathecae, if bears three not kidney-shape (**15**)….....….....……7

7. Eyes contiguous (**16**)……...…….8
Eyes separated (**17**)…….……..…....20

8. Third maxillary palp with a small or moderate rounded sensory pit with variable depth (**18**).....9
Third maxillary palp with various pits, variably in size and depth (**19**)…….....……....13

9. Eyes with inter-ommatidial pubescence (**20**). Small species with faint spot wings…..…
…...……..……..…….……..…....***C. chiopterus***
Eyes without pubescence (**21**) or slightly pubescent. Different characteristics.…...….....10

10. Antennal sensilla coeloconica pattern on segments 3, 12 to 15 (sometimes also in segment 11). Wing spots pattern well-distinct and clear. Cell R_5 with a distinct diabolo-shaped dark spot (**22**)……..…..……..……***C. imicola***
Antennal sensilla coeloconica pattern on segments 3, 11 to 15. Wing spots pattern indistinct. Without that spot or with another shape (**23**)……...…….11

11. Blurred wing spots (**24**). Spermathecae size unequal (**25**). Triangle defined by the three sensilla chaetica up the eye with isosceles triangle-shaped (**26**).…….***C. dewulfi***
More distinct wing spots but also blurred (**27**). Spermathecae mostly equal in size (**28**). Triangle defined by the three sensilla chaetica up the eye with equilateral triangle- shaped (**29**)……..……...…12

12. Spermathecae in length (Mean 60 μm). Chitinous plates width (Mean 41 μm). Third segment of the maxillary palp skewed and elongated, slender with the greatest width near apex (**30**).Wing length near 1,28 mm..……..……..…..***C. scoticus***
Spermathecae in length (Mean 50 μm). Chitinous plates width (Mean 33 μm). Third segment of the maxillary palp short and swollen, with the greatest width at the distal third (**31**).Wing length near 1,14 mm……...…….....***C. obsoletus***

13. Presence of cibarial ornamentation (**32**). Without dark spot in cubital cell…..….....14
Absence of cibarial ornamentation. Usually with dark spot in cubital cell or indistinct…15

14. Eyes joined for a long distance (more than 2 facets), (**33**). Third maxillary palp narrower in sub-apical region.…….……..…....***C. subfagineus***
Eyes joined for short distance (1 or 2 facets), (**34**). Third maxillary palp less narrower in sub-apical region……..……...***C. fagineus***

15. Tips of veins m_1, m_2 and cu_1 pale (**35**)…….....…16
Tips of veins m_1, m_2 and cu_1 dark (**36**)…….…..….....18

16. Cell R_5 with a narrow dark band-shaped spot. Pale tips of the veins m_1, m_2 and cu indistinct (**37**)……..……...***C. flavipulicaris***
Cell R_5 with a dark conspicuous diabolo-shaped spot. Pale tips of the veins m_1, m_2 and cu distinct (**38**)…….. …….…….17

17. Wing with a dark spot well-developed covering the basal part of vein m_2 and frequently joining veins m_2 and m_1 (**39**). Wing with conspicuous spots..…….***C. newsteadi***
Wing with a dark spot poor-developed covering only a small area of basal part of vein m_2 (**40**). Wing with small spots…….……...……...***C. punctatus***

18. Wing frequently without dark spot in cubital cell or with a trace of it. Occasional specimens with sensilla coeloconica absent in one segment from 11 to 13, usually absent in 12 (**41**). Vaguely-marked specimens (**42**)……..……..***C. impunctatus***
Wing with a dark spot in cubital cell (**43**). Sensilla coeloconica on segments 3, 11 to 15. Well-marked specimens…..……...……..……...….……19

19. Wing with dark spots distinct and small (**44**). Anal cell with two dark punctiform spots ……..……....***C. pulicaris***
Wing with dark spots indistinct and large (**45**). Anal cell with single indistinct dark spot. …...…….…….…….……..***C. lupicaris***

20. Conspicuous cibarial ornamentation (**46**)…….……....***C. heliophilus***
Without cibarial ornamentation……..……...…….21

21. Pale wings without spots, or with blurred dark spots (**47**)……..…...…...…22
Grey or brownish wings with spots pattern variable (**48**)……..…..…...…27

22. Eyes strongly pubescent (**49**)…….……***C. comosioculatus***
Eyes with short pubescence or no pubescent…….…..……..……23

23. Antennae with sensilla coeloconica on segments 3 to 10. Basal segments spherical, narrower than larger (**50**). Third palpal segment thick (**51**). Eyes widely separated (**52**). .....***C. minutissimus***
Different characteristics ……..…….……..……24

24. Sensilla coeloconica on segments 3, 7, 9, 13 to 15 and variably in 5 and 12 …..…… ……..…….…..***C. paradisionensis***
Different characteristics ……..…….……..……...……25

25. Sensilla coeloconica on segments 3, 13 to 15. Small-sized specimens. Maxillae without tooth (**53**). Spots pattern indistinct-blurred (**54**)..…….***C. pseudoheliophilus***
Sensilla coeloconica on segments 3, 11 to 15. Large-moderate sized specimens. Maxillae with tooth (**55**). With or without spots pattern ……..……..……..26

26. Wing with three small dark spots: one covering area below radial cells, other on vein cu_2 and other minute on distal part of vein cu_1 (**56**). Third segment of maxillary palp with some small closely-spaced sensory pits bordering a large pit (**57**). Antennal segments 9 and 10 dichromatic (**58**)…….……..……..……***C. brunnicans***
Wing without dark spots (**59**). Third segment of the maxillary palp with a large pit with irregular margins (**60**). Antennal segments 9 and 10 monochromatic (**61**) …...…***C. vexans***

27. Second radial cell (R_2) covered by a pale spot (**62**)……..……...……28
Second radial cell (R_2) entirely dark, (**63**) or only partly pale (**64**) ……..…...…….…30

28. Antennae without sensilla coeloconica on segments 4 to 10 and from 11 to 15 sensilla coeloconica atrophied (**65**). Third maxillary palp thin with one to various sensory pits (**66**). Wing with pale spots indistinct (**67**). Basal cell without macrotrichia……...…..***C. poperinghensis***
Antennal segments with typical well-developed sensilla coeloconica on segments 3 to 15 (**68**). Third maxillary palp thick with a large pit (**69**). Wing with pale spots distinct (**70-71**). Basal cell with macrotrichia….....…...…….…..…29

29. Pale apical spots of cells M_1, M_2 and Cu not rounded, cut by wing margin (**70**)…….. ……..……...***C. pictipennis***
Pale apical spots of cells M_1, M_2 and Cu prominent rounded, not cut by wing margin…..... (**71**)…….……..... ***C. univitattus***

30. Antennae without sensilla coeloconica on segments 11 to 15……..……...31
Antennae with sensilla coeloconica in all or some segments from 11 to 15….……....32

31. Wing with spots in distal parts of cells R_5, M_1, M_2, Cu and An (**72**). Third maxillary palp thick with a large sensory pit of well-defined margins and little deep (**73**). Post-pharyngeal ornamentation consist on one or two conspicuous spines (**74**)……. .....***C. longipennis***
Wing without spots in distal parts of R_5, M_1, M_2 but present in Cu and An (**75**). Third maxillary palp thick with a characteristic large and very deep sensory pit (**76**). Without characteristic ornamentation.……..…….…***C. semimaculatus***

32. Wing with pale spots in distal parts of cells R_5, M_1, M_2 (**77**)…….…...…..33
Wing without pale spots in distal parts of cells R_5, M_1, M_2 (**78**)…(except *C. picturatus* with some pale spots in margins but sensilla coeloconica distributed on segments 3, 11 to 15)…….....…43

33. Sensilla coeloconica on segments 3, 5, 7, 9 and 11 to 15…..…….…...…***C. truncorum***
Other sensilla pattern…….…….…43

34. Antennal segments without sensilla coeloconica on segments 11 to 15…….....…35
Antennal segments with sensilla coeloconica on 3 to 15 or 3 to 14…….....…36

35. Post-pharyngeal ornamentation consists on minute scattered points (**79**)……. ……..…..***C. shaklawensis***
Post-pharyngeal ornamentation consists on 5-8 conspicuous spines (**80**)…....***C. clastrieri***

36. Medial part of vein m_1 with a small scattered pale spot (**81**). Post-pharyngeal ornamentation consists on 3-5 spines (**82**)……....***C. festivipennis***
Medial part of vein m_1 without that pale spot. No post-pharyngeal ornamentation……...37

37. Sensilla coeloconica on segments 4 to 14 (**83**). Sensory pit with irregular margins (**84**)…….…….……..……..…….…....***C. griseidorsum***
Sensilla coeloconica on segments 3 to 15, variably in segment 10. Sensory pit spherical with regular margins……..…38

38. Basal part of cell M_1 with an isolated pale spot (**85**)……...……..…...…39
Basal part of cell M_1 without an isolated pale spot (**86**)……..…….……...…...…42

39. Basal cell of wing with macrotrichia (**87**)…….……..…40
Basal cell without macrotrichia (**88**)…….……...……..…..…41

40. Eyes almost contiguous [(0,5-1 facet of separation (**89**)]. Sensory pit very deep (**90**). Pale spot in basal part of cell M_1 situated not distal to pale spot adjoining cell R_2 distally, but under it (**91**). Moderately-sized wing.…….……...***C. duddingstoni***
Eyes separated by more than 1 facet (**92**). Sensory pit shallow (**93**). Pale spot in basal part of cell M_1 situated distal to pale spot adjoining cell R_2 distally (**94**). Largely-sized wing……..……..……..***C. maritimus***

41. Macrotrichia very abundant below the basal cell. Distal spots on wing margin moderately-defined (**95**). Other doubtful taxonomic characters: sensilla coeloconica on segment 10 and spermathecae duct with sclerotized ring…….……...***C. gejgelensis***
Macrotrichia abundant below the basal cell. Distal spots on wing margin very well-defined (**96**). Other doubtful taxonomic characters: without sensilla coeloconica on segment 10 and sclerotized ring.……..…….…...***C. cataneii***

42. Large pale spot covers area 1, vein r-m and continued anteriorly across M to basal part of M_2 (**97**). Pale spot in area 2 and R_5 large. Eyes moderately-separated (more than 1 facet) (**98**)..…….……...***C. simulator***
Small pale spot with well-defined margins, covers area 1 and vein r-m to M (area 1 and r-m spot is frequently separated from M spot) (**99**). Pale spot in area 2 and R_5 large small. Eyes slightly separated (less than 0,5 facets) (**100**).…….....……...***C. alazanicus***

43. Sensilla coeloconica ***at least*** on segments 3 and 7 to 14. Third segment of maxillary palp very thick with a well-defined sensory pit (**101**)……..……..…….....……..44
Sensilla coeloconica pattern and maxillary palp (**102**) otherwise……..…….....46

44. Sensilla coeloconica on segments 3, 5 to 14 (**103**). Occasional specimens with sensilla absent also in segments 5 and/or 6 in one or both antennae. Two indistinct pale spots in basal margins of cells M_1 and M_2 (**104**).....……..…***C. kurensis* cf.**
Sensilla coeloconica present on segments 3 to 14, variably in segment 15. Without spots in cells M_1 and M_2 …….……...…..…45

45. Sensilla coeloconica on segments 3 to 14. Third maxillary palp very thick and dark-brownish (specimens in alcohol). Palpal sensory pit with a characteristic aspect (**105**). Spermathecae oval-shape heavily sclerotized (**106**)……..…….….***C. odiatus***
Sensilla coeloconica on segments 3 to 15. Third maxillary palp less inflated and light-brownish (specimens in alcohol). Palpal sensory pit large with a typical aspect (**102**). Spermathecae piriform-shape moderately sclerotized (**107**)..…….……***C. kibunensis***

46. Sensilla coeloconica on segment 3 and on one or various segments from 4 to 10.....47
Sensilla coeloconica exclusively distributed on segments 3 and 11 to 15….…...…….….49

47. Sensilla coeloconica on segments 3, 5, 7, 9, 10 to 14, variably in 5 and 6. Wing on brownish background with indistinct pale spots distributed across the entire wing (**108**). Third maxillary-palp with pit bearing very irregular margins, sometimes with small-pits bordering a main pit (**109**)…….....……..……..…..***C. santonicus***
Other characteristics……...……48

48. Sensilla coeloconica on segments 3, 7, 9, 13 to 15, occasionally also in 5 and 12. Wing light-brownish background with indistinct pale spots on r-m and area 2 (**110**). Third maxillary palp with a single pit with well-defined margins (**111**)……..……...
……..…***C. paradisionensis***
Sensilla coeloconica on segments 3 and 11 to 15 but variable, sometimes with single or various segments from 7 to 10 also with sensilla coeloconica (**112**). Large dark-brownish wing with pale spots on r-m and area 2 (**113**). Third maxillary palp subcylindrical-shape with a single sensory pit bordered by various small ones (**114**)……
….....***C. furcillatus***

49. Two spines in the fourth tarsomere of medial legs (**115**). Brownish wing. Large-sized specimens (2>mm)…….…50
Absence of spines in the fourth tarsomere of medial legs (**116**). Brownish or light-brownish wing.. Medium-sized specimens (1,5 mm average)……..…....51

50. Wing with two pale spots, arranged at area r-m and over area 2. Absence of spots in cells M_2 and An (**117**). Moderately brownish and pubescent wing. Basal cell without macrotrichia…….....***C. achrayi***
Wing with three pale spots, arranged over vein r-m, extending over cell M (sometimes forming two separated spots) and in cell An and area 2 (**118**). Strongly brownish and pubescent wing on anterior margins. Basal cell with abundant macrotrichia…….
….……..….....***C. fascipennis***

51. Brownish wings with pale spots in cells An, Cu, M_2, M_1 and also frequently in R_5…..
(**119**)……..……***C. picturatus***
Light-brownish wing with without pale spots in cells M_2, M_1 and R_5.....52

52. Wing without spots in cells An and Cu. Wings with indistinct pale spots, one on vein r-m and other one on area 2 (**120**)..……..……...…….***C. pallidicornis***
Wing with indistinct spots in cells An, Cu and rarely on M_2 (**121**). Wings with distinct pale spots, one on vein r-m and other one on area.……..….....***C. subfasciipennis***

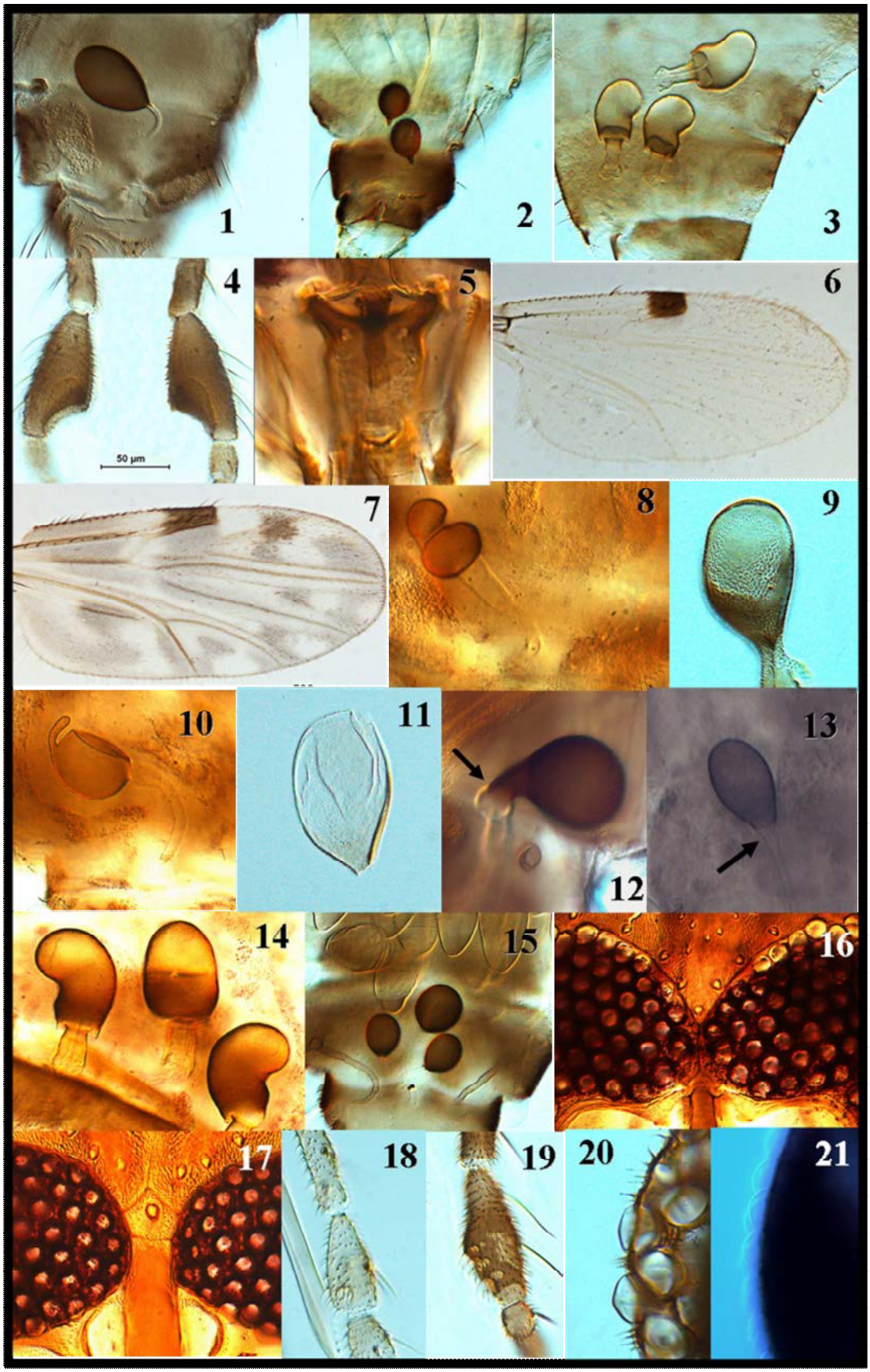


Plate key 1: (1) *C. circumscriptus* (spermatheca); (2) *C. obsoletus* (spermathecae); (3) *C. tauricus* (spermathecae); (4) *C. circumscriptus* (palpus), (5) (ornamentation); (6) *C. parroti* (wing); (7) *C. nubeculosus* (wing); (8) *C. parroti* (spermatheca), (9) (halters); (10) *C. stigma* (spermatheca), (11) (halters); *C. nubeculosus* (spermatheca, arrow pointing neck); (13) *C. riethi* (spermatheca, arrow pointing spermatheca neck); (14) *C. tauricus* (spermathecae); (15) *C. festivipennis* (spermathecae, aberrant size); (16) *C. imicola* (eyes); (17) *C. santonicus* (eyes); (18) *C. dewulfi* (palp); (19) *C. newsteadi* (palp); (20) *C. chiopterus* (facets); (21) *C. lupicaris* (facets).

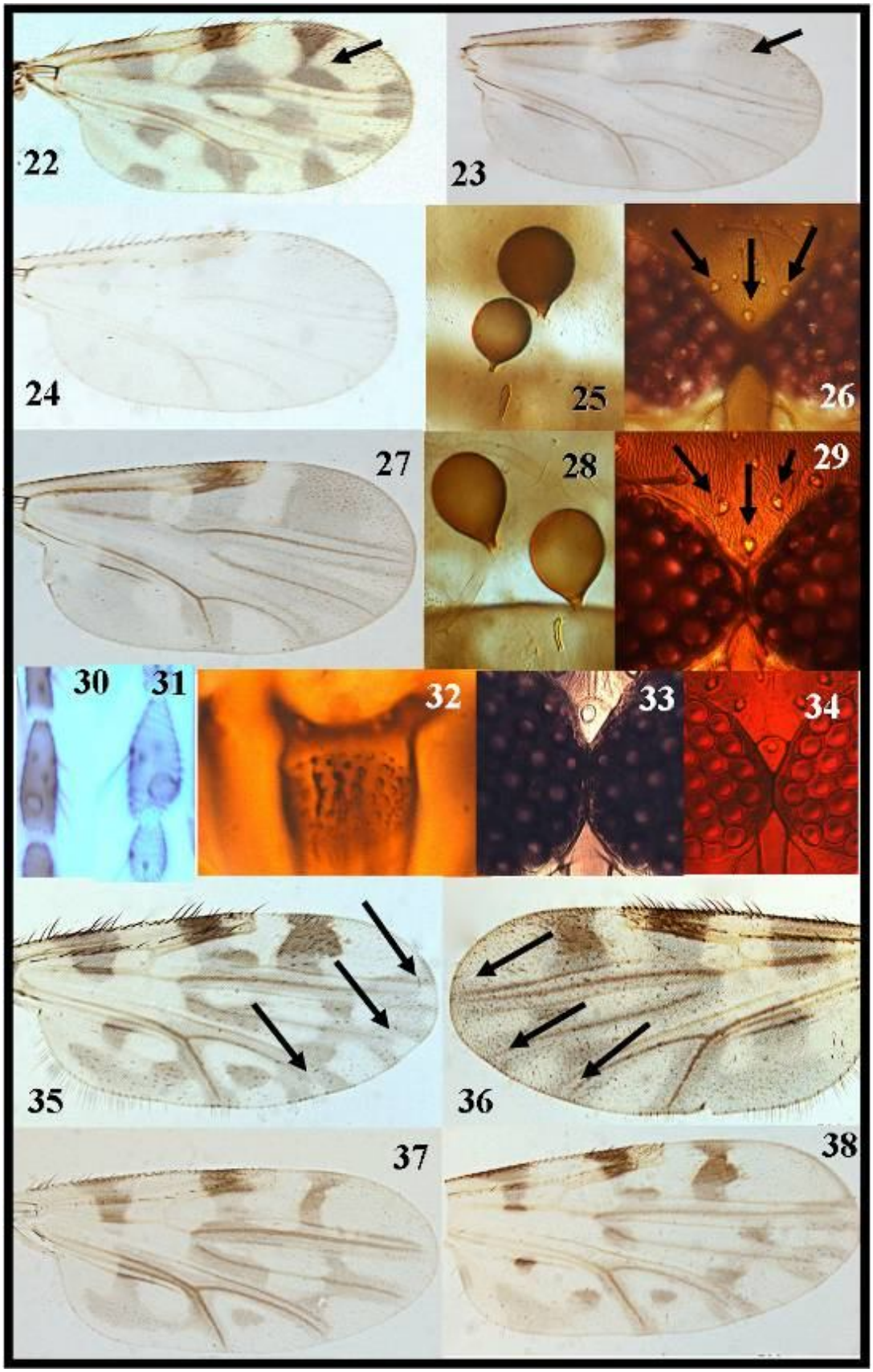


Plate key 2: (22) *C. imicola* (wing, arrow pointing R_5 spot shape); (23) *C. obsoletus* (wing, arrow pointing R_5 spot shape); (24) *C. dewulfi* (wing), (25) (spermathecae); (26) (eyes, arrows pointing supraorbital setae); (27) *C. scoticus* (wing), (28) (spermathecae), (29) (eyes, arrows pointing supraorbital setae); (30) *C. scoticus* (palp); (31) *C. obsoletus* (palp); (32) *C. fagineus* (cibarial ornamentation); (33) *C. subfagineus* (eyes); (34) *C. fagineus* (eyes); (35) *C. newsteadi* (wing, arrows pointing tips of veins m_1, m_2 and cu_1); (36) *C. lupicaris* (wing, arrows pointing tips of veins m_1, m_2 and cu_1); (37) *C. flavipulicaris* (wing); (38) *C. punctatus* (wing).

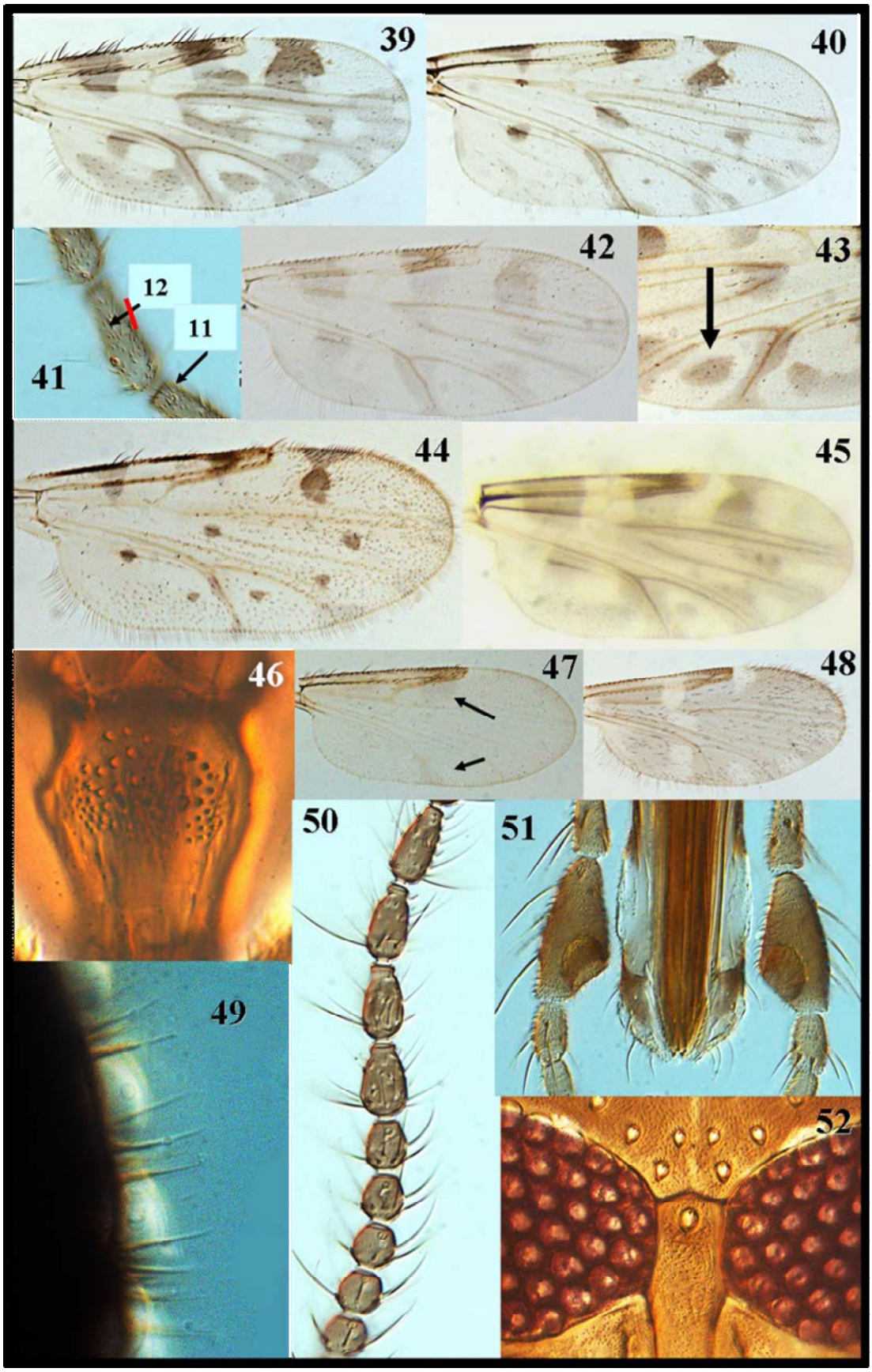


Plate key 3: (39) *C. newsteadi* (wing); (40) *C. punctatus* (wing); (41) *C. impunctatus* (antennal segments), (42) (wing); (43) *C. lupicaris* (wing, arrow pointing cubital cell wing spot); (44) *C. punctatus* (wing); (45) *C. lupicaris* (wing); (46) *C. heliophilus* (cibarial ornamentation); (47) *C. brunnicans* (wing, arrows pointing blurred dark spots); (48) *C. alazanicus* (wing); (49) *C. comosioculatus* (facets); (50) *C. minutissimus* (antennal segments), (51) (palpus), (52) (eyes).

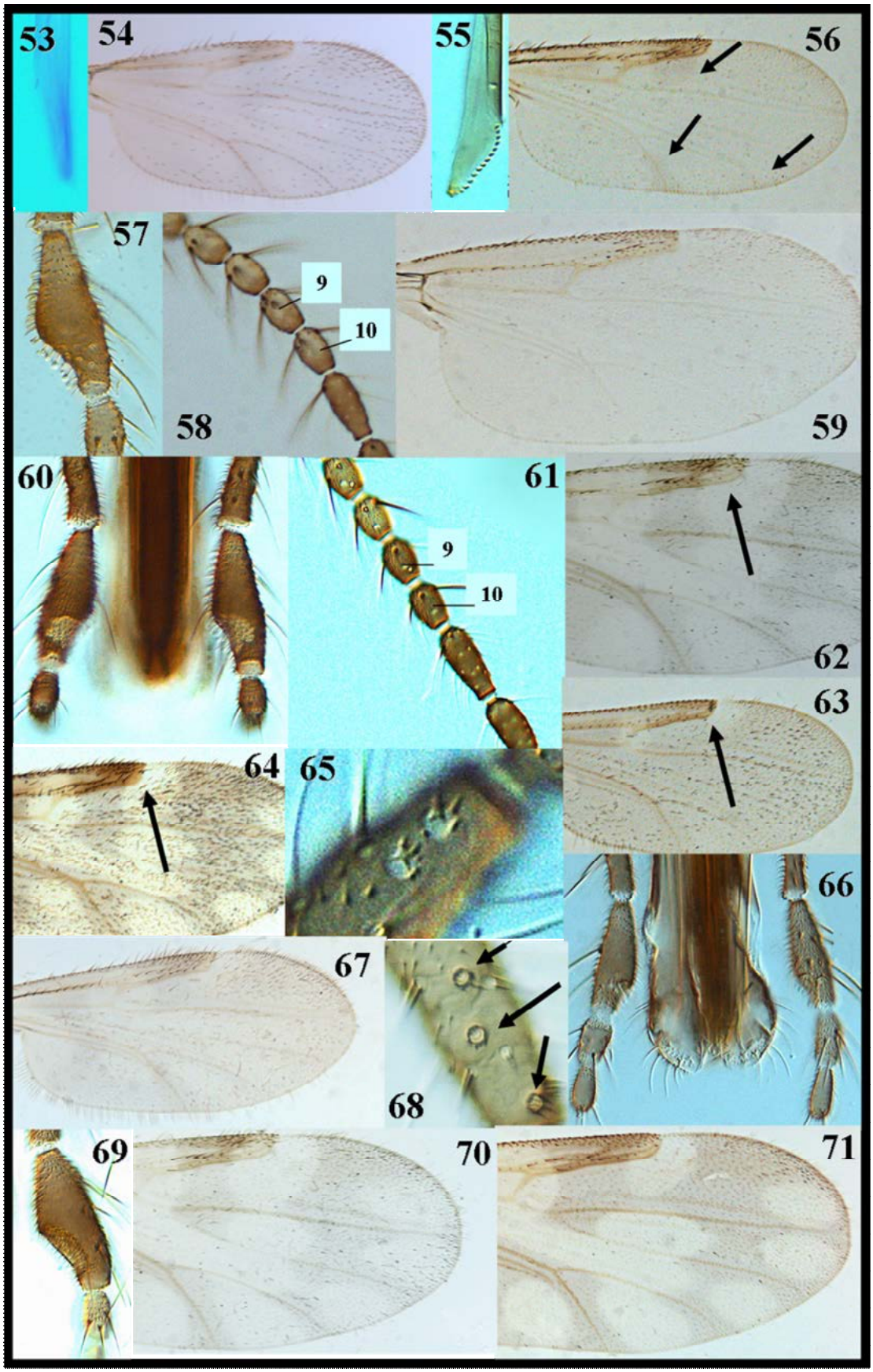


Plate key 4: (53) *C. pseudoheliophilus* (maxilla), (54) (wing); (55) *C. brunnicans* (maxilla), (56) (wing, arrows pointing blurred dark spots), (57) (palp), (58) (antennal segments); (59) *C. vexans* (wing), (60) (palpus), (61) (antennal segments); (62) *C. pictipennis* (wing, arrow pointing second radial cell spot); (63) *C. kibunensis* (wing, arrow pointing second radial cell spot); (64) *C. gejgelensis* (wing, arrow pointing second radial cell spot); (65) *C. poperinghensis* (sensilla coeloconica), (66) (palpus), (67) (wing); (68) *C. pictipennis* (antennal segment, arrows pointing typical aspect of sensilla coeloconica), (69) (palp); (70) *C. pictipennis* (distal part of the wing); (71) *C. univitattus* (distal part of the wing).

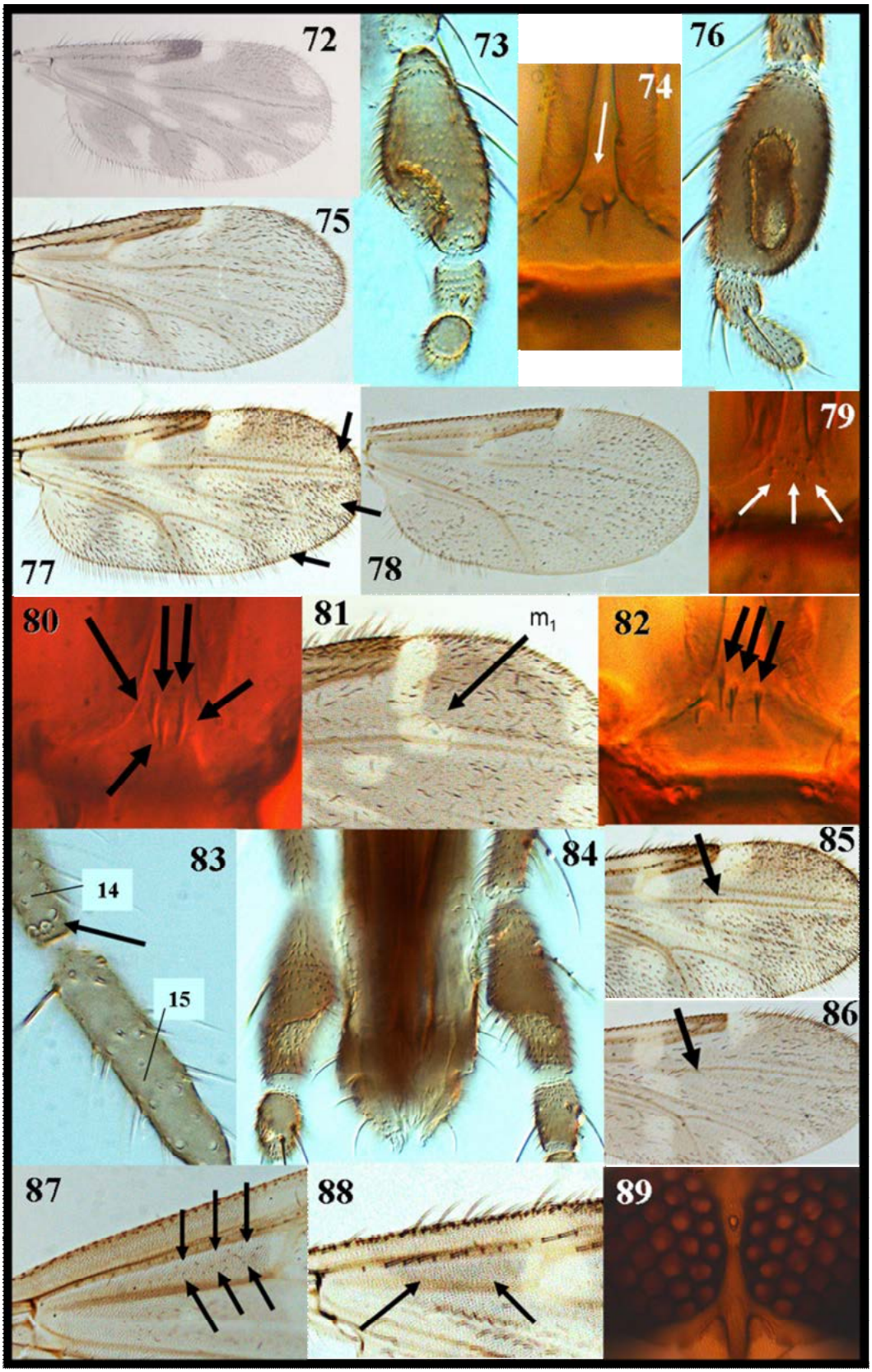


Plate key 5: (72) *C. longipennis* (wing), (73) (palp); (74) (ornamentation, arrow pointing spines); (75) *C. semimaculatus* (wing), (76) (palp); (77) *C. gejgelensis* (wing, arrows pointing tips spots of cells R_5, M_1 and M_2); (78) *C. kibunensis* (wing); (79) *C. shaklawensis* (ornamentation, arrow pointing punctiform structures); (80) *C. clastrieri* (ornamentation, arrows pointing spines); (81) *C. festivipennis* (wing, arrow pointing m_1 spot); (82) (ornamentation, arrows pointing spines); (83) *C. griseidorsum* (distal antennal segments, arrow pointing sensilla coeloconica), (84) (palpus); (85) *C. cataneii* (wing, arrow pointing cell M_1); (86) *C. alazanicus* (wing, arrow pointing absence of M_1 spot); (87); *C. duddingstoni* (wing, arrows pointing macrotrichae on basal cell); (88) *C. cataneii* (wing, arrow pointing basall cell without macrotrichae); (89) *C. duddingstoni* (eyes).

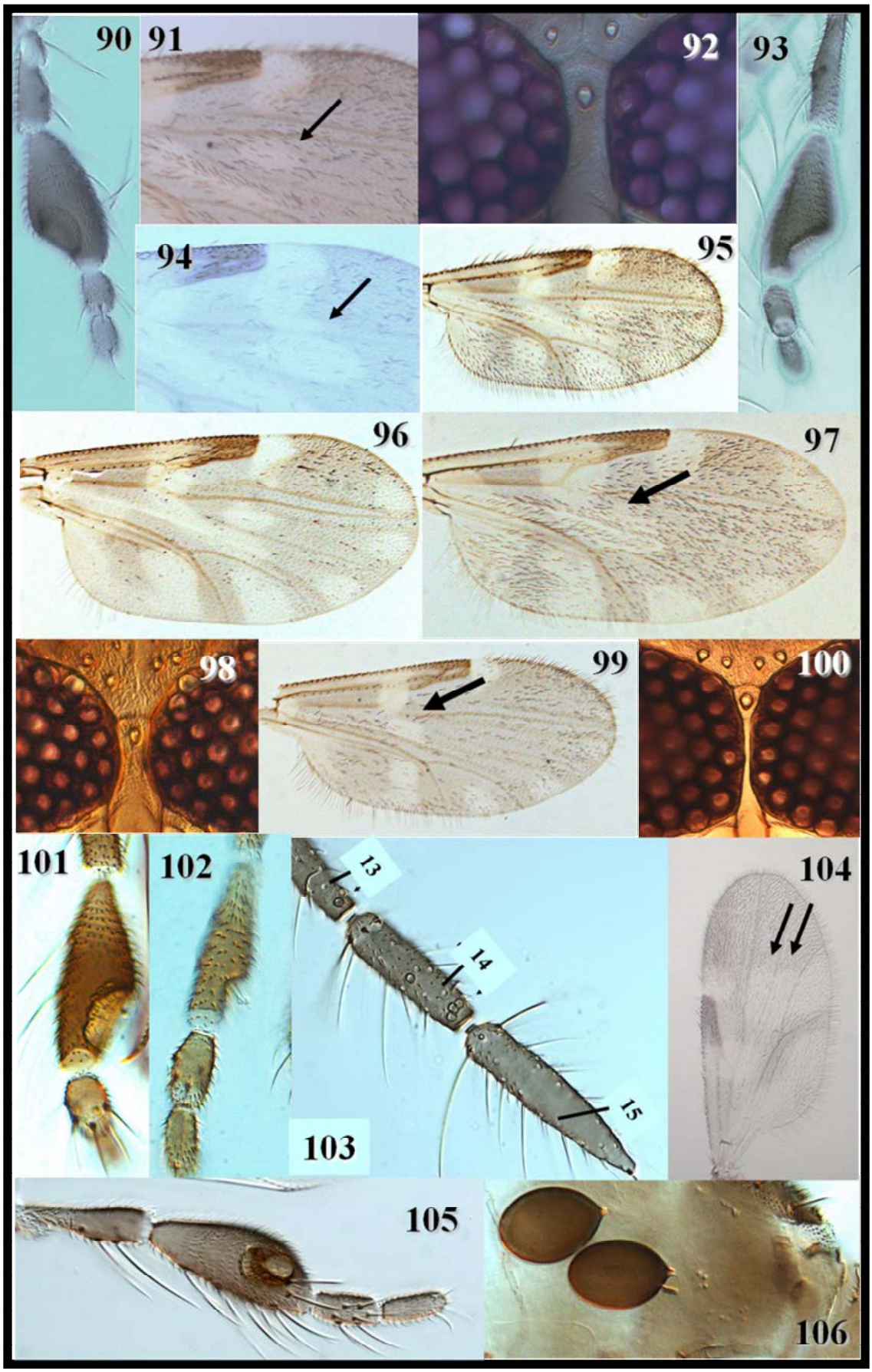


Plate key 6: (90) *C. duddingstoni* (palp), (91) (wing, arrow pointing spot position on M_1); (92) *C. maritimus* (eyes), (93) (palp); (94) (wing, arrow pointing spot position on M_1); (95) *C. gejgelensis* (wing); (96) *C. cataneii* (wing); (97) *C. simulator* (wing, arrow pointing M-M_2 spot size), (98) (eyes); (99) *C. alazanicus* (wing, arrow pointing M spot size), (100) (eyes); (101) *C. kibunensis* (palp); (102) *C. furcillatus* (palp); (103) *C. kurensis* (antennal distal segments), (104) (wing, arrows pointing pale spots), (105) (palp), (106) (spermathecae).

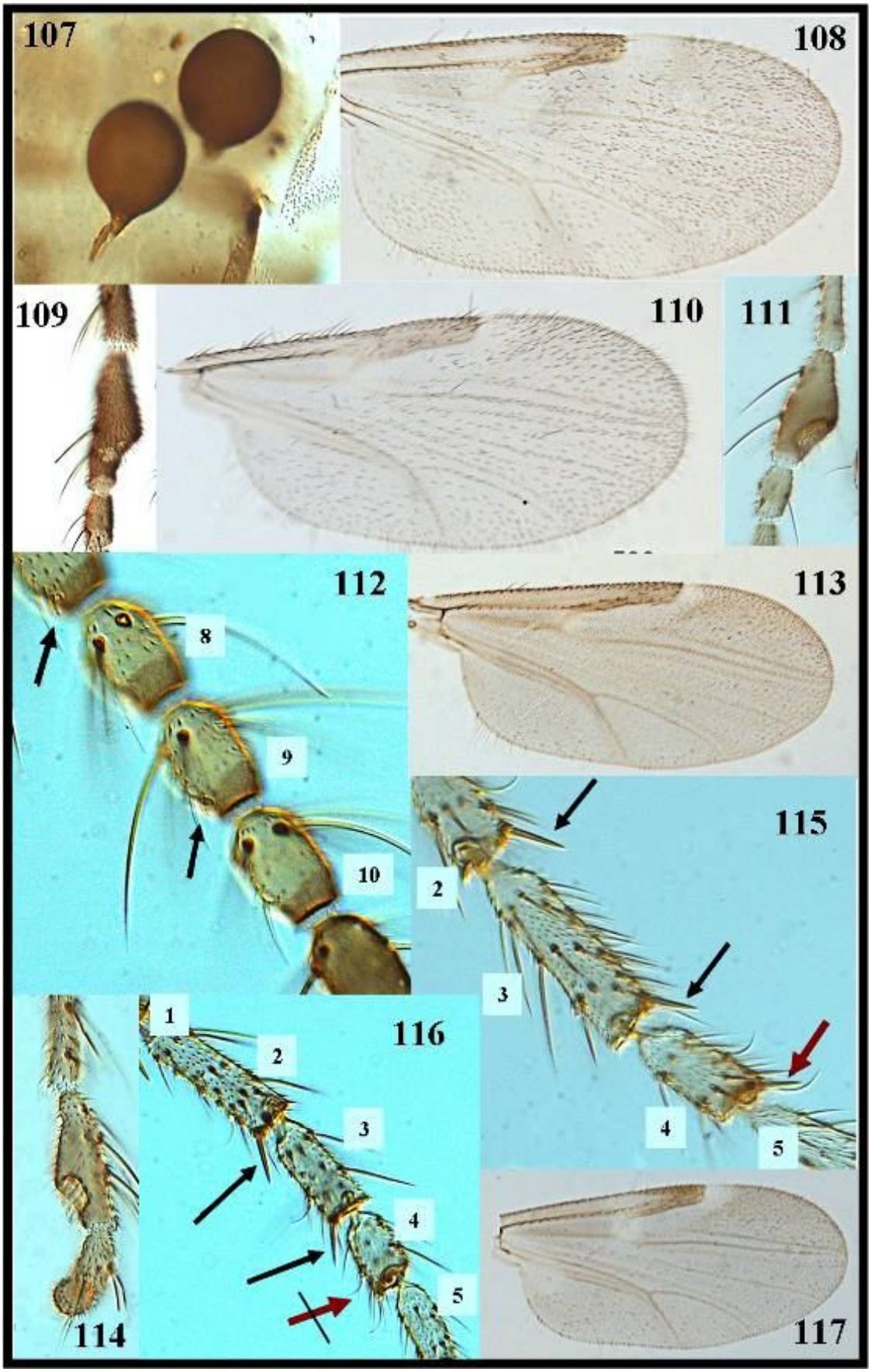


Plate key 7: (107) *C. kibunensis* (spermathecae); (108) *C. santonicus* (wing), (109) (palp); (110) *C. paradisionensis* (wing), (111) (palp); (112) *C. furcillatus* (antennal segments, arrow pointing sensilla coeloconica), (113) (wing), (114) (palp); (115) *C. achrayi* (leg, arrows showing right-side spines of tarsomeres); (116) *C. pallidicornis* (leg, arrows showing spines of tarsomeres and absence of spines in fourth tarsomere); (117) *C. achrayi* (wing).

Plate key 8: (118) *C. fascipennis* (wing); (119) *C. picturatus* (wing); (120) *C. pallidicornis* (wing); (121) *C. subfascipennis* (wing).

5.5. Dichotomous key for *Culicoides* males identification (slide-mounted specimens)
Key based on Delécolle (1985) and Glukhova (2005)

1. Parameres completely fused near middle (**1**) or basal region…….…….…….…….....2
Parameres entirely separated, or rarely fused at base by a thin imperceptible chitinous suture (**2**)…..……..……..……..……..…5

2. Wing with single conspicuous dark spot on second radial cell, contrasting, light background (**3**)……..……..……..……..…….....3
Wing with evident spot pattern……...…….……...……..……...4

3. Ninth tergite with two prominent lobes (**4**). Membrane of ninth sternite spiculate (**5**)….
..……..…...…*C. stigma*
Ninth tergite without lobes (**6**). Membrane of ninth sternite bare (**7**).…….…….....***C. parroti***

4. Aedeagus broad and square-shaped with apical part ending in a concave notch (**8**)…...
……..…….....***C. nubeculosus***
Aedeagus more or less triangle-shaped with apical part divided in two pointed processes (**9**)……..……*C. riethi*

5. Inner surface of basistyle with a characteristic setulose area (thicked, shorted and cogged setae), (**10**)……...…….....……...……...…….…..…..6
Inner surface of basistyle without that characteristic setulose area or if bears, with simple aspect……...…..……..……12

6. Wing without dark spot on cubital cell (**11)**. Well-marked specimens.……..…..…...
……..…..…..***C. fagineus/C. subfagineus***
Wing with dark spot on cubital cell or traces of it, if not bad-marked specimens…….....7

7. Tips of veins m_1, m_2 and cu_1 pale (**12**)……...……...……..……8
Tips of veins m_1, m_2 and cu_1 dark (**13**)…….……...…..……..10

8. Cell R_5 with a narrow dark band-shaped spot. Pale tips of the veins m_1, m_2 and cu indistinct (**14**)…...……..……....…*C. flavipulicaris*
Cell R_5 with a dark conspicuous diabolo-shaped spot. Pale tips of the veins m_1, m_2 and cu distinct (**15**)…….……..……..…….…….9

9. Wing with a dark spot well-developed covering the basal part of vein m_2 and frequently joining veins m_2 and m_1 (**16**)……...……..…..……..*C. newsteadi*
Wing with a dark spot poor-developed covering only a small area of basal part of vein m_2 (**17**)…….……..……..*C. punctatus*

10. Without sensilla coeloconica in segment 13. Wing frequently without dark spot in cubital cell or with a trace of it. Ventral root of basistyle poor-developed, inconspicuous. Vaguely-marked specimens…….....……...*C. impunctatus*
Frequently with sensilla in segment 13. Wing with a dark spot in cubital cell. Ventral root of basistyle conspicuous and pointed. Well-marked specimens….…….……..11

11. Wing with dark spots distinct and small (**18**). Anal cell with two dark punctiform spots ……..……...…...*C. pulicaris*
Wing with dark spots indistinct and large (**19**). Anal cell with single indistinct dark spot. …...…….……..……..……...*C. lupicaris*

12. Basistyle with ventral root (**20**)……....13
Basistyle with ventral and dorsal root (**21**)……..……...…....14

13. Wing with distinct pale spots (**22**). Membrane of ninth sternite spiculate (**23**). Eyes not pubescent ……....***C. circumscriptus***
Wing without spots. Membrane of ninth sternite bare. Eyes strongly pubescent (**24**)…..... ……..…….……..***C. comosioculatus***

14. Light wings without spots, or with blurred dark spots (**25, 26**)……..……..…...…15
Grey or brownish wings with spots pattern variably (**22**)…..……..……...…..…...…..…21

15. Parameres stick-shaped, thick and more or less straight along the entire length or slightly curved (**27**). Apicolateral processes very slender, long with pointed tips (**28**)……. ……..…….....***C. tauricus***
Parameres and apicolateral processes otherwise……...……16

16. Ventral root of basistyle foot-shaped (**29**). Median or basal part of aedeagus with two minute lateral appendices (**30**)…….……..……....17
Ventral root of basistyle simple (**31**). Median or basal part of aedeagus without lateral appendices……...……..……..…18

17. Wing without spots. Tergite with two prominent lobes (**32**). Parameres slender and crossed. Ventral process of ventral root of basistyle bad-developed (**33**)…….....***C. vexans***
Wing with some blurred dark spots. Tergite without lobes. Parameres thick and curved gradually into a S-bend (**34**).Ventral process of ventral root of basistyle well-developed ……..……..……...***C. brunnicans***

18. Aedeagus Y-inverted shaped heavily sclerotized (**35**)…..…….....***C. paradisionensis***
Aedeagus otherwise, median process of aedeagus quadrangular and triangular; almost small……..…..…..…19

19. Parameres crossed with a comb structure on tips (**36**). Ninth tergite without notch (**37**). Membrane of ninth sternite often with few spicules (**36**)..….....…***C. minutissimus***
Parameres not crossed and without additional structures. Ninth tergite with a minute notch. Membrane of ninth sternite completely bare……...……...…….20

20. Apicolateral processes well-developed. Median process of aedeagus large, longer than broader (**38**)…….……...***C. pseudoheliophilus***
Apicolateral processes poor-developed. Median process of aedeagus small, as broad as long (**39**) ……...……...…..***C. heliophilus***

21. Ninth tergite without apicolateral processes or if bears, with small rounded prominences. Antennae with segment 13 bearing a single sensilla chaetica in medial part and 2-3 in basal part……...…22
Ninth tergite with apicolateral processes of variable size. Distribution of sensilla chaetica different…….…….....…26

22. Eyes with inter-ommatidial pubescence. Ninth tergite with two small apicolateral processes (**40**). Membrane of ninth sternite bare (**41**).....…….....***C. chiopterus***
Eyes without pubescence or slightly pubescent. Ninth tergite without developed apicolateral processes. Membrane of ninth sternite bare or spiculate….....…..23

23. Ninth sternite completely divided in two independent lobes. Aedeagus strongly sclerotized and strengthened (**42**)…….……...…***C. scoticus***
Ninth sternite with a cleft but not separated in two parts. Aedeagus with lateral arms forming a trapeziform arch weakly sclerotized. If not, aedeagus moderately sclerotized and less conspicuous…….…….....24

24. Aedeagus without lateral arms forming a trapeziform arch. Ninth sternite with a prominent cleft (**43**). Membrane of ninth sternite bare…….……...….....***C. obsoletus***
Aedeagus with lateral arms forming a trapeziform arch (**44**). Ninth sternite with caudomedian excavation concave. Membrane of ninth sternite spiculate ……...…25

25. Ninth tergite with two wide, rounded and shorted apicolateral processes (**45**). Ninth tergite margins of smooth surface. Wing spots pattern well-distinct and clear.....
……..…***C. imicola***
Ninth tergite without processes and with scaly surface margins (**46**). Wing spots pattern indistinct and blurred.…….……...……***C. dewulfi***

26. Membrane of ninth sternite bare (**47**)…….…..27
Membrane of ninth sternite spiculate (**48**)……..……...……..…….....42

27. Second radial cell (R_2) covered by a pale spot (**49**)……..……...….28
Second radial cell (R_2) entirely dark, (**50**) or only partly pale……..……..…....…30

28. Apicolateral processes slender (**51**). Ninth tergite with inconspicuous notch. Parameres fused at base by a thin imperceptible chitinous suture (**52**). Median process of aedeagus rectangular or trapezoidal, short-medium length (**53**)...……...***C. poperinghensis***
Apicolateral processes thick. Ninth tergite with a notch. Parameres completely separated. Median process of aedeagus rectangular, long length (**54**)……29

29. Pale apical spots of cells M_1, M_2 and Cu not rounded (cut by wing margin)…….…..…
…….……***C. pictipennis***
Pale apical spots of cells M_1, M_2 and Cu prominent rounded (not cut by wing margin)…...
……..…….. ***C. univittatus***

30. Parameres with a serrated structure, comb-shaped (**55**)……...…31
Parameres without that structure (**56**)…….....32

31. Wing without spots in distal parts of cells R_5, M_1, M_2. Median process of aedeagus with two prominent appendices…….....……..***C. semimaculatus***
Wing with spots in distal parts of cells R_5, M_1, M_2. Median process of aedeagus different…….……..……..….***C. longipennis***

32. Wing without pale spots in distal parts of cells R_5, M_1, M_2 (**57**) ……...….....33
Wing with pale spots in distal parts of cells R_5, M_1, M_2, sometimes blurred ….....…34

33. Apicolateral processes widely separated. Ventral root short. Median process of aedeagus trapezoidal-shaped (**58**)…….……..…….***C. kurensis***
Apicolateral processes moderately separated. Ventral root long. Median process of aedeagus rectangular-shaped (**59, 60**)…..…...…….....***C. kibunensis***

34. Ventral roots foot-shaped (**61, 62**)……..…….……..…..…35
Ventral roots simple…….…..36

35. Bad-marked specimens, with pale indistinct spots in entire wing (**63**). Parameres curved gradually into a S-bend. Medial process of aedeagus rectangular and conspicuous with two minute lateral appendices (**64**)……..……***C. santonicus***
Well-marked specimens, with pale spots in all apical margins (**65**). Parameres different. Medial process of aedeagus triangular without lateral appendices (**66**)…..…..***C. truncorum***

36. Basal part of cell M_1 not covered by a pale spot……...……..…….37
Basal part of cell M_1 covered by a pale spot……..38

37. Large pale spot covers area 1, vein r-m and continued anteriorly across M to basal part of M_2. Pale spot in area 2 and R_5 large (**6**7). Medial process of aedeagus with a swollen side in sub-apical region (**68**).....……..…….…..……..……..…….....***C. simulator***
Small pale spot with well-defined margins, covers area 1 and vein r-m to M (area 1 and r-m spot is frequently separated from M spot). Pale spot in area 2 and R_5 small (**69**). Medial process of aedeagus straight, without a pronounced side (**70**).....***C. alazanicus***

38. Median process of aedeagus triangular with a pointed projection (**71**). Very well-marked and distinct wing pattern spots (**72**)…..……..***C. shaklawensis***
Different characteristics……...……..……39

39. Medial part of vein m_1 with a characteristic pale spot (**73**). Presence of post-pharyngeal ornamentation ……...……..…40
Medial part of vein m_1 without such pale spot. Absence of such ornamentation…….....41

40. Wing with three scattered pale spot or a single trilobulate spot in cell M. R_5 pale spot usually not reaching area 2 (**73**)..…..……....***C. festivipennis***
Wing with a large non-stop single pale spot in cell M. R_5 pale spot usually larger reaching area 2 (**74**)……..……..……...***C. clastrieri***

41. Medial process of aedeagus with a sub-apical narrowing (**75**)..……....***C. gejgelensis***
Medial process of aedeagus without a sub-apical narrowing (**76**)……..…….....***C. cataneii***

42. Wing with pale spots in distal parts of cells R_5, M_1, M_2 ……...…….....…...…43
Wing without pale spots in distal parts of cells R_5, M_1, M_2 ……..…….45

43. Membrane of ninth sternite with few spicules. Medial process of aedeagus rectangular (**77**)……...***C. duddingstoni***
Membrane of ninth sternite spiculate or strongly spiculate. Medial process of aedeagus variable in shape ……..…….……...……...…..….44

44. Apex of aedeagus convex (rounded), (**78**)…..……...……...…….***C. griseidorsum***
Apex of aedeagus slightly convex (mostly truncated), (**79**)……...……..***C. maritimus***

45. Apicolateral processes long and pointed (**80**). Ninth tergite without notch. Medial process of aedeagus rectangular (**81**). Membrane of ninth sternite with few spicules (**83**) ……..…….…….....***C. furcillatus***
Apicolateral processes medium-sized and rounded. Ninth tergite with a deep median notch (**82**). Medial process of aedeagus solid and trapezoidal. Membrane of ninth sternite spiculate (**83**)……...…….....…….....….46

46. Ditistyle swollen in the median region and apex sharped (**84**). Membrane of ninth sternite with few spicules……...……....***C. odiatus***

Ditistyle not swollen at median region and apex normal (**85**). Membrane of ninth sternite strongly spiculate……..……..47

47. Ventral roots short (**86**)…….……..……..48
Ventral roots long (**87**)…….....……49

48. Dark-brownish wing (**88**). Presence of two spines in fourth tarsomere of medial legs……. ……..……..***C. fascipennis***
Light-brownish wing (**89**). Absence of two spines in fourth tarsomere of medial legs…..……..……..…….…….....***C. pallidicornis***

49. Wing without pale spots in cell An and Cu (**90**). Presence of two spines in fourth tarsomere of medial legs…….……....….***C. achrayi***
Wing with pale spots in cell An and Cu (very indistinct in *C. subfasciipennis*). Absence of two spines in fourth tarsomere of medial legs……...……...….50

50. Wing with blurred pale spots in cells An and Cu……..…….***C. subfasciipennis***
Wing with pale spots in cells An, Cu, M_2, M_1 and sometimes in R_5 (**91**)……..
……..…***C. picturatus***

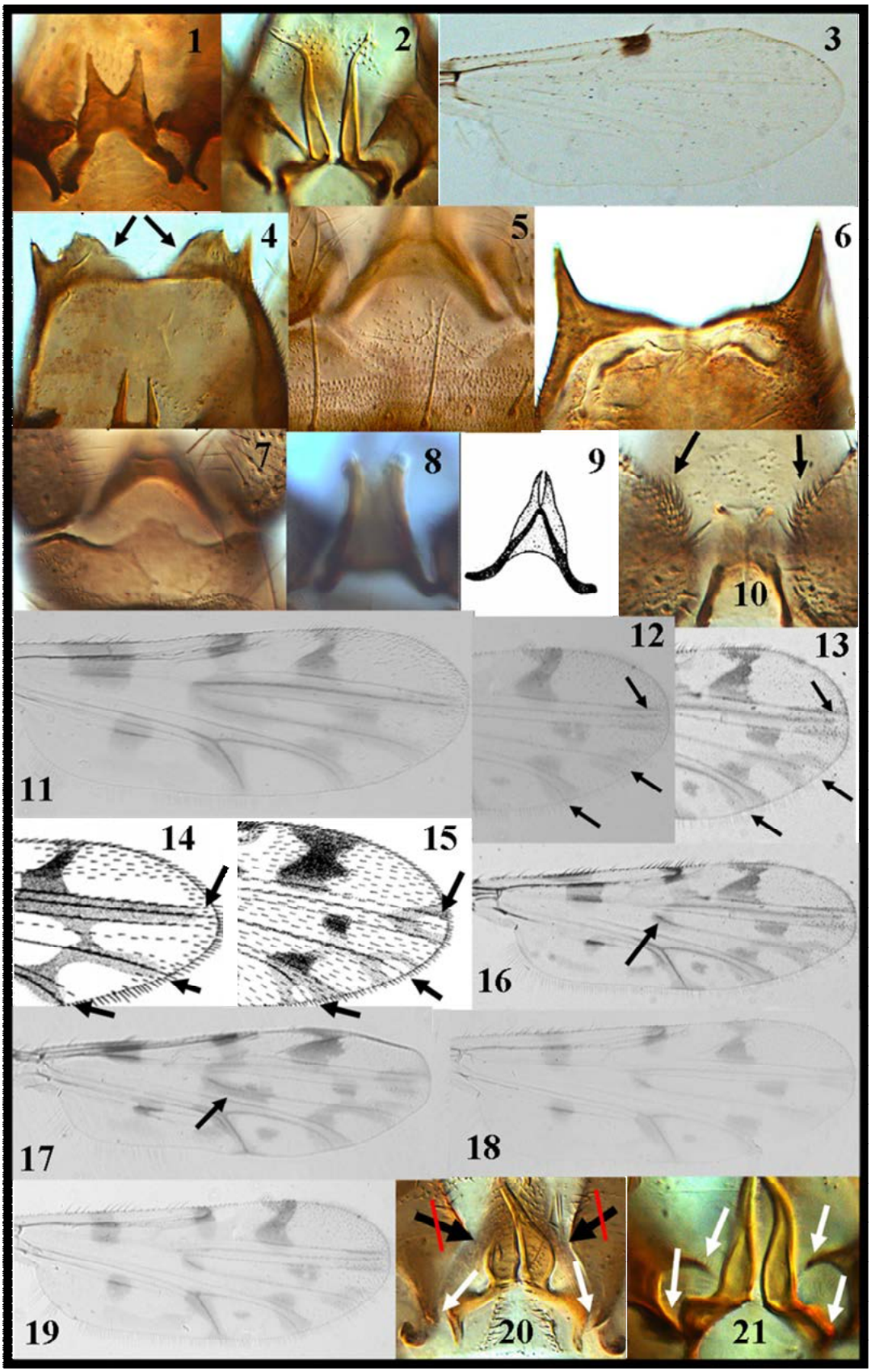


Plate key 1: (1) *C. parroti* (parameres); (2) *C. gejgelensis* (parameres); (3) *C. parroti* (wing); (4) *C. stigma* (ninth tergite, arrows pointing lobes); (5) *C. stigma* (membrane of ninth sternite), (6) (apicolateral processes); *C. parroti* (7) (membrane of ninth sternite); (8) *C. nubeculosus* (aedeagus); (9) *C. riethi* (aedeagus); (10) *C. pulicaris* (basistyle, arrows pointing setulose area); (11) *C. fagineus* (wing); (12) *C. lupicaris* (wing, arrow pointing tips of veins m_1, m_2 and cu_1); (13) *C. punctatus* (wing, arrows pointing tips of veins m_1, m_2 and cu_1); (14) *C. flavipulicaris* (wing, arrows pointing tips of veins m_1, m_2 and cu_1); (15) *C. punctatus* (wing, arrows pointing tips of veins m_1, m_2 and cu_1)); (16) *C. punctatus* (wing, arrow pointing the characteristic spot); (17) *C. newsteadi* (wing, arrow pointing the characteristic spot); (18) *C. pulicaris* (wing); (19) *C. lupicaris* (wing); (20) *C. circumscriptus* (basistyle roots, arrows pointing ventral roots); (21) *Culicoides* spp. (basistyle roots, arrows pointing ventral and dorsal roots).

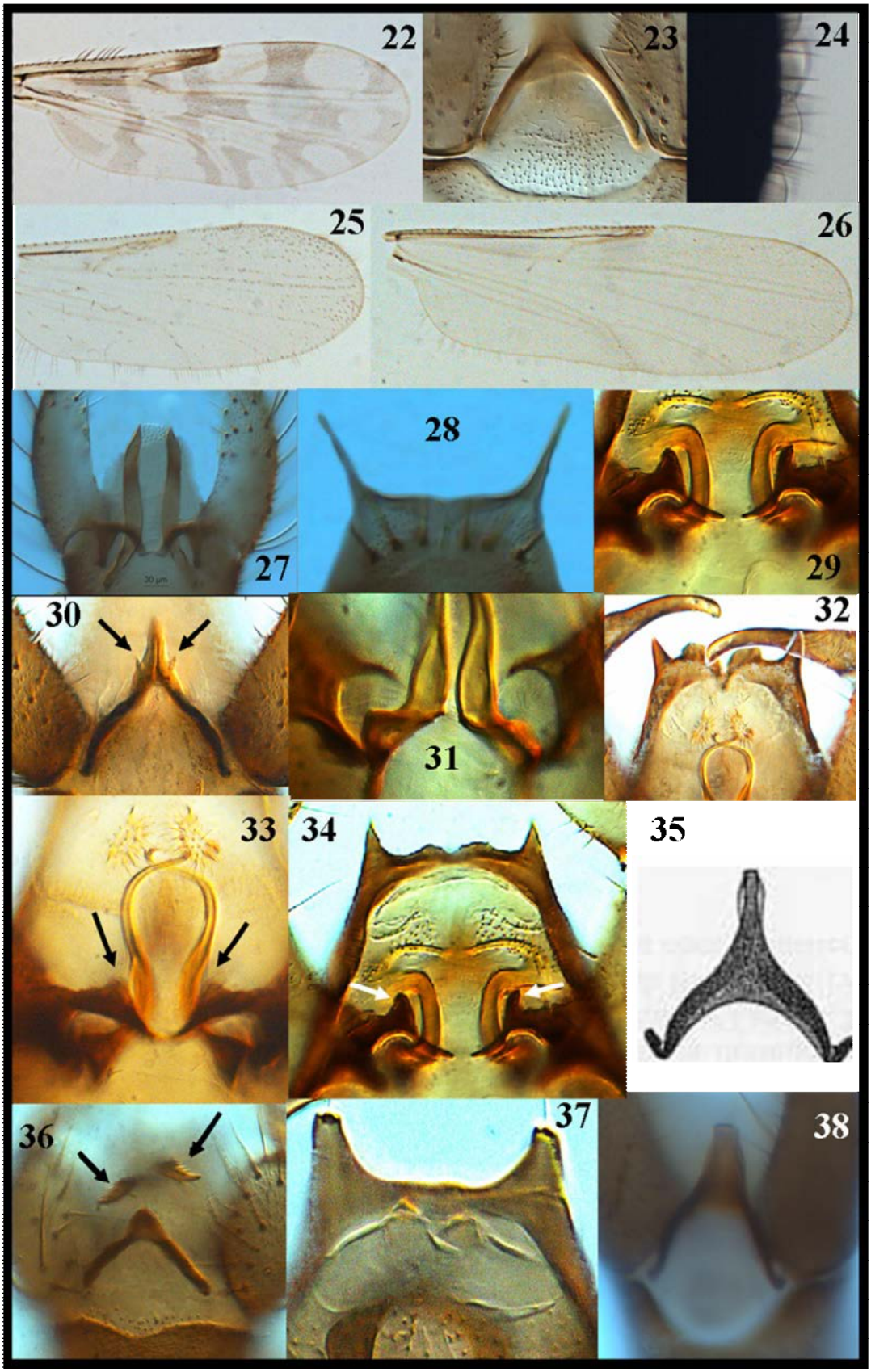


Plate key 2: (22) *C. circumscriptus* (wing), (23) (membrane of ninth sternite); (24) *C. comosioculatus* (facets of eyes); (25) *C. tauricus* (wing); (26) *C. brunnicans* (wing); (27) *C. tauricus* (parameres), (28) (apicolateral processes); (29) *C. brunnicans* (ventral roots); (30) *C. vexans* (aedeagus, arrows showing lateral appendices); (31) *Culicoides* spp. (ventral roots); (32) *C. vexans* (lobes of ninth tergite), (33) (parameres and roots, arrows pointing ventral roots); (34) *C. brunnicans* (parameres and roots, arrows pointing ventral roots); (35) *C. paradisionensis* (aedeagus); (36) *C. minutissimus* (parameres, arrows pointing tips), (37) (ninth tergite), (38) *C. pseudoheliophilus* (aedeagus).

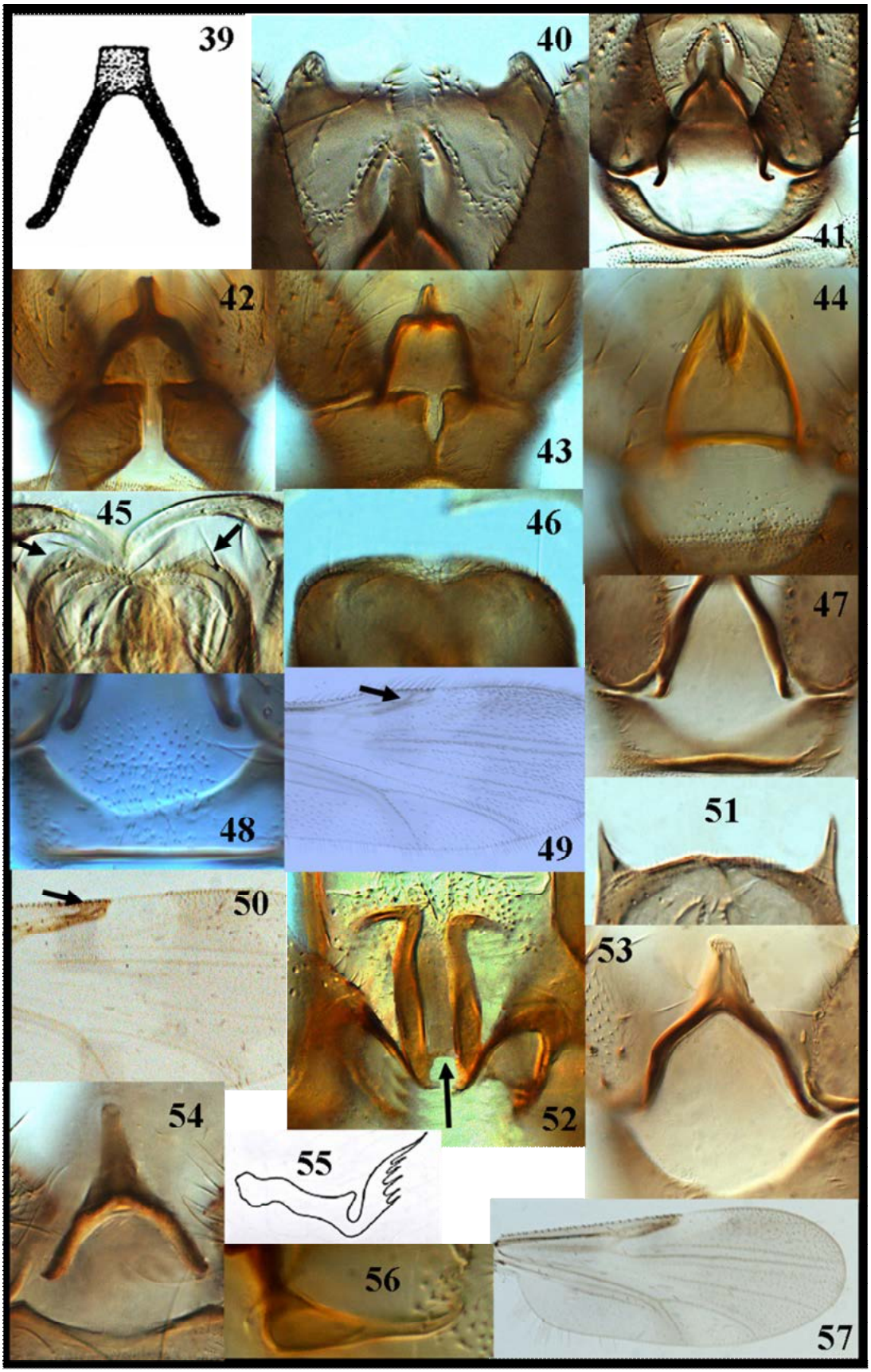


Plate key 3: (39) *C. heliophilus* (aedeagus); (40) *C. chiopterus* (apicolateral processes); (41) (membrane of ninth sternite); (42) *C. scoticus* (ninth sternite and aedeagus); (43) *C. obsoletus* (ninth sternite and aedeagus); (44) *C. imicola* (aedeagus), (45) (ninth tergite, arrows pointing apicolateral processes); (46) *C. dewulfi* (hind surface of ninth tergite); (47) *C. poperinghensis* (membrane of ninth sternite); (48) *Culicoides* spp. (membrane of ninth sternite); (49) *C. pictipennis* (wing, arrow pointing second radial cell); (50) *C. simulator* (wing, arrow pointing second radial cell); (51) *C. poperinghensis* (apicolateral processes), (52) (parameres, arrow indicating imperceptible suture), (53) (aedeagus); (54) *C. pictipennis* (aedeagus); (55) *C. longipennis* (paramere); (56) *Culicoides* spp. (paramere); (57) *C. kibunensis* (wing).

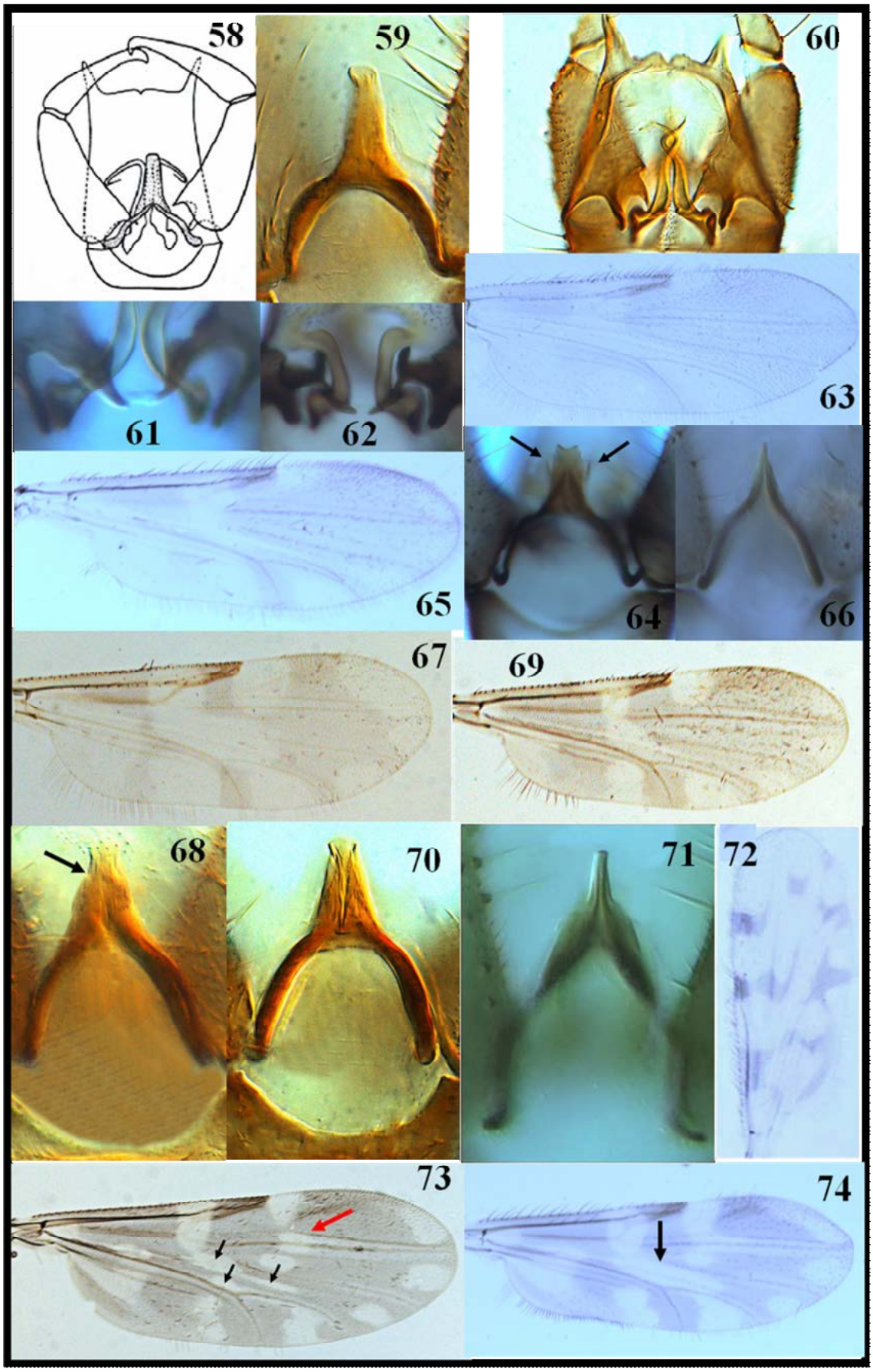


Plate key 4: (58) *C. kurensis* (genitalia); (59) *C. kibunensis* (aedeagus), (60) (genitalia); (61) *C. truncorum* (ventral roots); (62) *C. santonicus* (ventral roots), (63) (wing), (64) (aedeagus, arrows pointing lateral appendices); (65) *C. truncorum* (wing), (66) (aedegaus); (67) *C. simulator* (wing), (68) (aedeagus, arrow pointing swollen side); (69) *C. alazanicus* (wing); (70) (aedeagus); (71) *C. shaklawensis* (aedeagus), (72) (wing); (73) *C. festivipennis* (wing, black arrows pointing the characteristic spots distribution and red arrow pointing m_1 spot); (74) *C. clastrieri* (wing, arrow pointing the large spot).

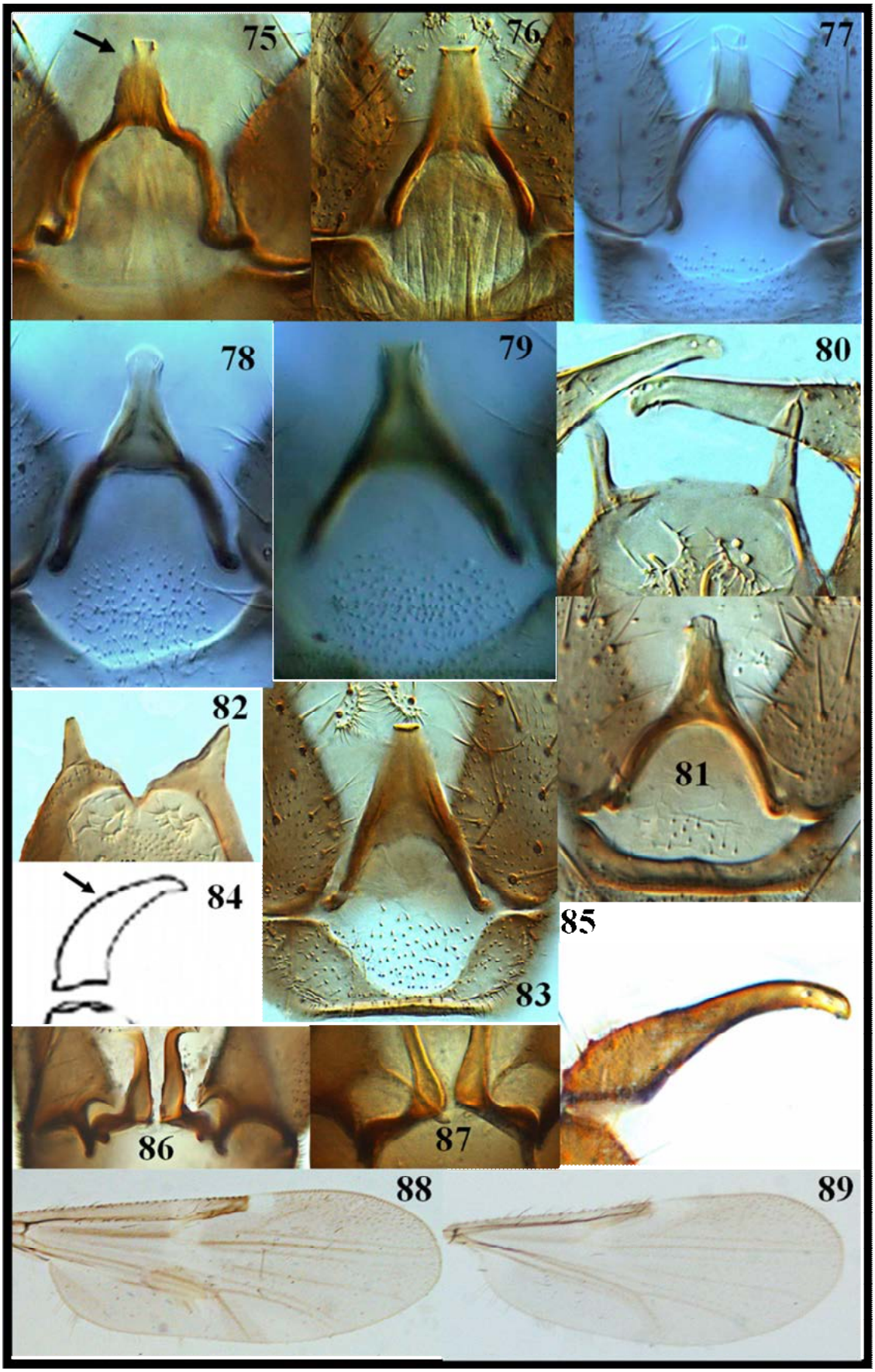


Plate key 5: (75) *C. gejgelensis* (aedeagus, arrow pointing sub-apical narrowing); (76) *C. cataneii* (membrane of ninth sternite and aedeagus); (77) *C. duddingstoni* (membrane of ninth sternite and aedeagus); (78) *C. griseidorsum* (membrane of ninth sternite and aedeagus); (79) *C. maritimus* (membrane of ninth sternite and aedeagus); (80) *C. furcillatus* (apicolateral processes), (81) (membrane of ninth sternite and aedeagus); (82) *C. achrayi* (membrane of ninth tergite), (83) (membrane of ninth sternite and aedeagus); (84); *C. odiatus* (ditistyle, arrow pointing swollen side); (85) *C. fascipennis* (ditistyle); (86) *C. pallidicornis* (ventral roots); (87) *C. picturatus* (ventral roots); (88) *C. fascipennis* (wing); (89) *C. pallidicornis* (wing).

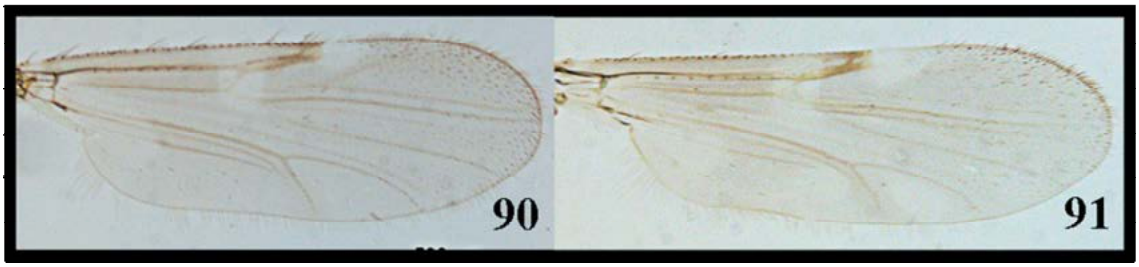


Plate key 6: (90) *C. achrayi* (wing); (91) *C. picturatus* (wing).

Note: Due to males are usually less attractive to light traps than females, some species of males have not been captured during the sampling. Therefore, these photographs have been obtained from other sources:

- Picture 9, 39 (Delécolle, 1985)
- Picture 55, 58 (Glukhova, 2005)
- Picture 14-15, 84 (Mathieu et al. 2010)
- Picture 35 (Delécolle et al. 2005)

5.6. Other ceratopogonid genera

In Spain, ceratopogonids comprise more than 210 species, belonging to 19 genera and grouped in four subfamilies: Forcipomyiinae with the genus *Forcipomyia* and *Atrichopogon*, Dasyheleinae with *Dasyhelea*, Ceratopogoninae includes the tribes Ceratopogonini, Culicoidini, Heteromyiini, Palpomyiini and Shaeromiini, and finally the subfamily Leptoconopinae with the genus *Leptoconops* (Delécolle, 2002). Taxa distribution of the family Ceratopogonidae in the Iberian Peninsula are shown in Figure 2.

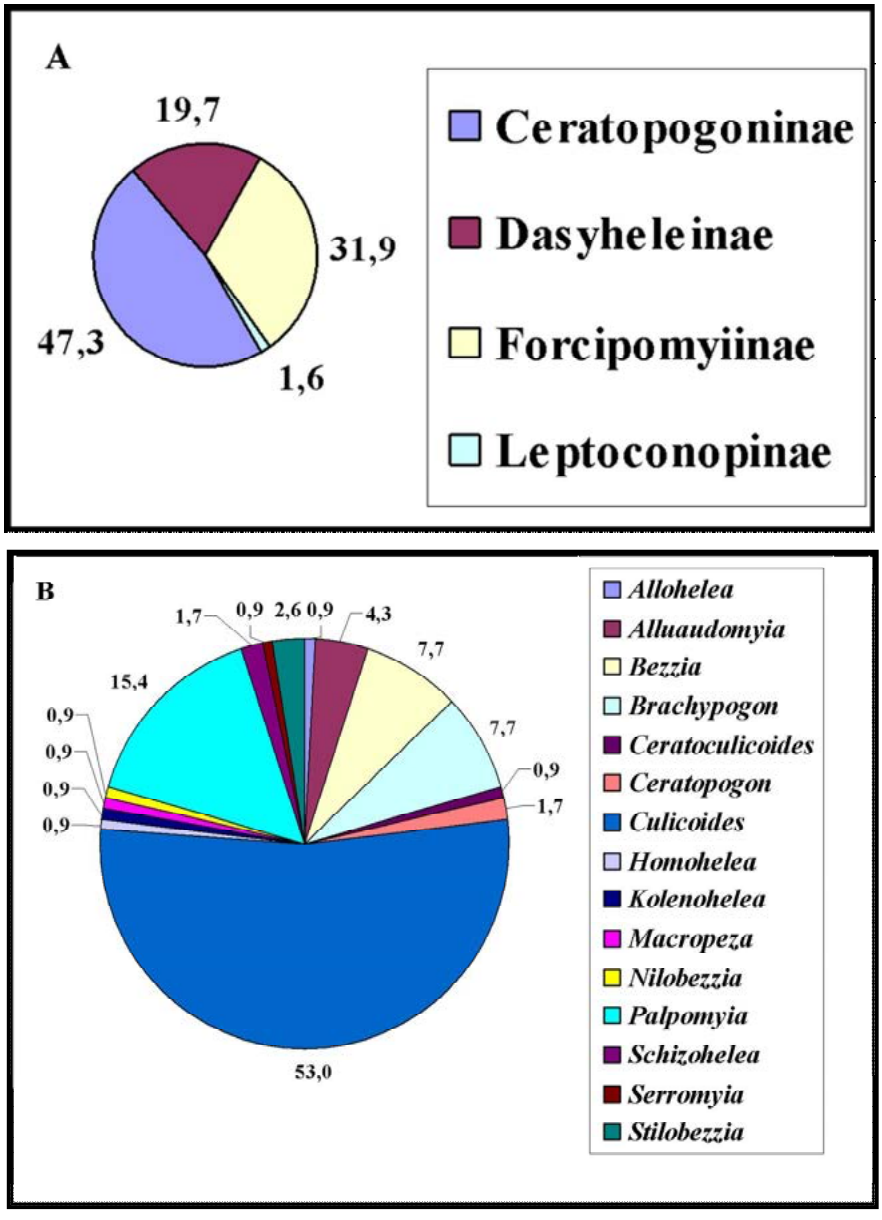


Fig. 2. (A) Percentage distribution of subfamilies from Ceratopogonidae family in the Iberian Peninsula **(B)** Percentage distribution of different genera of the subfamily Ceratopogoninae in the Iberian Peninsula.

Regarding the Basque Country, 15 different genera of Ceratopogonidae family (excluding genus *Culicoides*) have been accidentally trapped during different monitoring programs conducted between 2007-2013 (Table 1-2).

Table 1. List of Ceratopogonidae genera collected in sheep farms at the Basque Country using light traps.

		SHEEP FARMS			
	CERATOPOGONIDAE	**GIPUZKOA**	**ALAVA**	**BISCAY**	**TOTAL**
	Ceratopogininae				
1	*Allohelea tesellata*	+			**1**
2	*Bezzia* spp.		++		**1**
3	*Brachypogon* spp.		+		**1**
4	*Ceratoculicolides havelkai*	+		+	**2**
5	*Ceratopogon* spp.	+	+		**2**
6	*Homohelea iberica*				**0**
7	*Kolenohelea calcarata*	+	+		**2**
8	*Palpomyia* spp.			+	**1**
9	*Serromyia femorata*			+	**1**
10	*Monohelea* spp.	++	+		**2**
11	*Stilobezzia* spp.		+	++	**2**
	Dasyheleinae				
12	*Dasyhelea* spp.	+	+	+	**3**
	Forcipomyiinae				
13	*Forcipomyia* spp.	++++	++++	++++	**3**
14	*Atrichopogon* spp.	++	+++	++	**3**
	Leptoconopinae				
15	*Leptoconops noei* [a]		+		**1**
	Σ	**8**	**9**	**7**	**14**

[a] Captured with CDC traps without light sources and baited with dry ice (González et al. 2013c).
Symbols: +: 1-10; ++: 10-100; +++: 100-1000; ++++: > 10000 specimens.

Table 2. List of Ceratopogonidae genera collected in natural habitats at Alava province using light traps.

		NATURAL HABITATS							
	CERATOPOGONIDAE	**WETLAND**	**OAK GROVE**	**SHRUBLAND**	**PINE FOREST**	**ECOTONE**	**GRASSLAND**	**POND**	**TOTAL**
	Ceratopogininae								
1	*Allohelea tesellata*								**0**
2	*Bezzia* spp.					+	+		**2**
3	*Brachypogon* spp.				+				**1**
4	*Ceratoculicolides havelkai*								**0**
5	*Ceratopogon* spp.				+				**1**
6	*Homohelea iberica*						+		**1**
7	*Kolenohelea calcarata*								**0**
8	*Palpomyia* spp.						+	+	**2**
9	*Serromyia femorata*								**0**
10	*Monohelea* spp.								**0**
11	*Stilobezzia* spp.		+			+	+		**3**
	Dasyheleinae								
12	*Dasyhelea* spp.	+	+			+	+		**4**
	Forcipomyiinae								
13	*Forcipomyia* spp.	+++	+	++	++	++	+	++	**7**
14	*Atrichopogon* spp.	+	+	+	+	+	+	++	**7**
	Leptoconopinae								
15	*Leptoconops noei*								**0**
	Σ	**3**	**4**	**2**	**3**	**5**	**7**	**3**	**9**

Symbols: +: 1-10; ++: 10-100; +++: 100-1000; ++++: > 10000 specimens.

6. DISCUSSION

A key to all the *Culicoides* of the Basque Country has been developed, which involves 52 species. The poli-morphic characters used in the present key in order to differ between *Culicoides* species have been chosen by personal knowledge and criteria, helped by three main sources, Delécolle (1985), Glukhova (2005) and the interactive identification key of Mathieu et al. (2010). Due to the abundance of synonyms amongst *Culicoides* species, the nomenclature of Szadziewski and Borkent (2004) was followed.

Culicoides species have been checked and shown to be successfully identified using the characters described in the present dichotomous keys. Although some species are clearly easier to identify due to distinctive features, others could entail some difficulties. Indeed, the interpretation of some characters (sometimes subjective) require the observer's decision and therefore correct identification depends on the point of view and experience of the entomologist. In general, the use of stereomicroscopes for *Culicoides* identification is a useful tool to achieve a subgenera level identification but sometimes also serves as a species level identification (depending on binocular magnification, besides wing pattern, palps and other features are also possible to be

observed). However, badly-marked specimens are more problematic than well-marked specimens and usually require microscope observation for reliable and accurate identification (Mathieu et al. 2012). Although the key focuses on slide-mounted specimens, occasionally some features of specimens in alcohol are mentioned as additional clues. Indeed, fresh alcohol specimens usually show characteristic brightness and tones with immense value but which are lost when mounted.

Taxonomy and systematics in *Culicoides* biting midges, are usually referred to as an imbroglio, because of the enormous controversy in describing new sibling and/or cryptic species (Borkent, 2013). These difficulties are in particular more common in various species of *Avaritia* subgenera that are considered as sibling species based on the absence of female diagnostic characters whereas males exhibit substantial differences on their genitalia. This is the case of *C. obsoletus* and *C. scoticus* cryptic species (Garros et al. 2010). An attempt was made to separate each of the female species (*C. obsoletus* and *C. scoticus*) in the key, according to the new contributions of Augot et al. (2010), Nielsen and Kristensen (2011) and our own criteria.

On the other hand, female members of the subgenus *Culicoides* are slightly different in wing pattern morphology whereas the male genitalia is almost identical. The taxonomy within both subgenera (*Avaritia* and *Culicoides*) has been historically difficult in the absence of additional approaches. Although new molecular analyses have revealed new non-described cryptic species in Catalonia, Spain (Pagés et al. 2009). The different geographical distribution and climate in each region could be favorable to the apparition of new intraspecific variations in morphology in some species that could entail some discrepancies in the identification characters if the specimens do not come from the same area of study.

The complex Pulicaris that includes *C. pulicaris* and *C. lupicaris* (both members of the subgenera *Culicoides*) is also an example of two sibling species that entail difficulties. Even though they are easily distinguished due to their wing pattern (the latter has a blurred and indistinct spots pattern wing and a characteristic long dark spot on the anal cell) (Delécolle, 1985) there are various ranges of intraspecific variations on the wing pattern that usually seem to correspond with intermedian species between *C. pulicaris* and *C. lupicaris*. For instance, we have considered two different morphotypes in *C. lupicaris*, the nominal type Fig. 3B and an atypical type Fig. 3A based on their

size and wing spots pattern. Further molecular analysis to clarify the presence of new complexes of morphologically similar species are required.

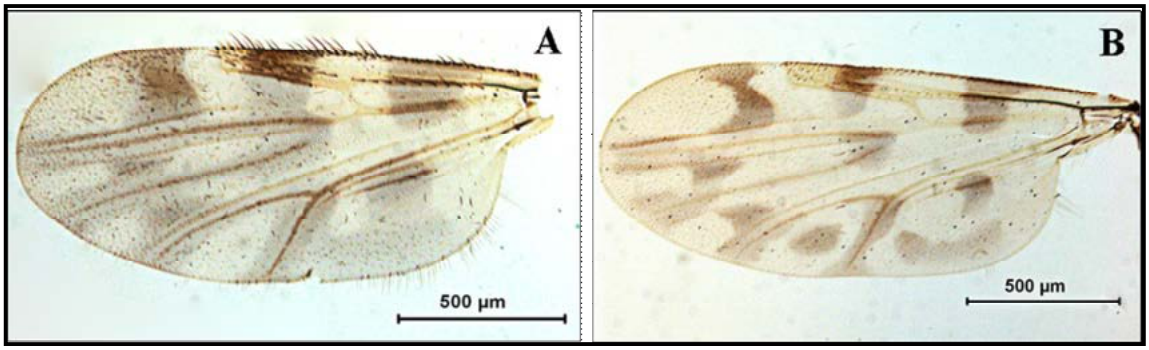


Fig. 3. Wing pattern of both morphotypes of *Culicoides lupicaris* found in the Basque Country **(A)** atypical species morphotype, pigmented with blurred pattern wing **(B)** nominal species, with clear and well-defined spots pattern.

Another question is the identification of both sexes of the sibling species *C. fagineus* and *C. subfagineus*. Talavera et al. (2010) revealed the presence of both cryptic sympatric species in Catalonia based on molecular analyses. It is most likely that both closely-related species coexist in the same areas but *C. fagineus* was more common than the latter. According to the descriptions of Delécolle (1985) and Delécolle and Ortega (1998), *C. fagineus*/*C. subfagineus* male species closely resemble *C. impunctatus*, but the hind margin of the ninth tergite of the males are remarkably convex and has a notch on the middle (it is straight or slightly concave in *C. impunctatus*). Very few male specimens have been collected in this thesis and these characters are not as clear as mentioned by these authors, and so we have used other diagnostic characters to separate both cryptic species for *C. impunctatus*. Indeed, males of *C. fagineus* and *C. subfagineus* have been pooled together.

Finally, we highlighted some new insights about the morphology of certain species. i.e. *C. picturatus* and *C. santonicus*. All of them are quite common in our region and showed important intraspecific variations. In the case of *C. picturatus* the wing pattern could be well-marked and distinct and other specimens showed indistinct pale margin spots. The body size, spots distribution and sensory pits of *C. santonicus* vary enormely among specimens. In our view, there are two clear morphotypes, the nominal with a length wing of: 1,5 mm and wide: 0,7 mm with with a single sensory pit of irregular margins and the atypical morphotype with 1-3 separated sensory pits and 1,75 mm length and 0,85 mm wide.

On the other hand, *Culicoides kurensis* is an uncommon species found in the Basque Country, with the sensilla coeloconica distribution on 3, 7-14, variable on 5-6. In contrast, captured specimens do not match with those reported in the literature (Glukhova, 1989; Mathieu et al. 2010). In which, the sensilla distribution is 3, 8-14 and variable in 7. The remaining morphological features are quite similar, except that the pale spots on the wing are less distinct in our specimens. Further studies, both morphological and molecular to elucidate if *C. kurensis* belongs to the same species or is a new morphotype or perhaps must to be separated as new species. Provisionaly, given the lack of other similar species we named it as *C. kurensis* cf.

7. CONCLUSIONS

The entomological monitoring program implemented in the present thesis enhances the knowledge concerning the *Culicoides* diversity in Basque Country. The substantial number of species recorded, both in permanent livestock areas (sheep farms) as well as in natural habitats (wildlife fauna) are of immense interest for understanding the presence/absence of species of interest as vector-borne transmissors.

This compilation is the first illustrated key from Spain and serves for the identification of a total of 52 taxa of *Culicoides* (Diptera: Ceratopogonidae) that comprises 65% of the total species recorded in Spain up to date.

The wealth of pictures is a great advantage for the training of taxonomists and is an important tool to help scientists in identifying species. The development of keys for arthropods involved in pathogen transmission is a valuable tool for entomologists and facilitate the dissemination of knowledge.

8. ACKNOWLEDGEMENTS

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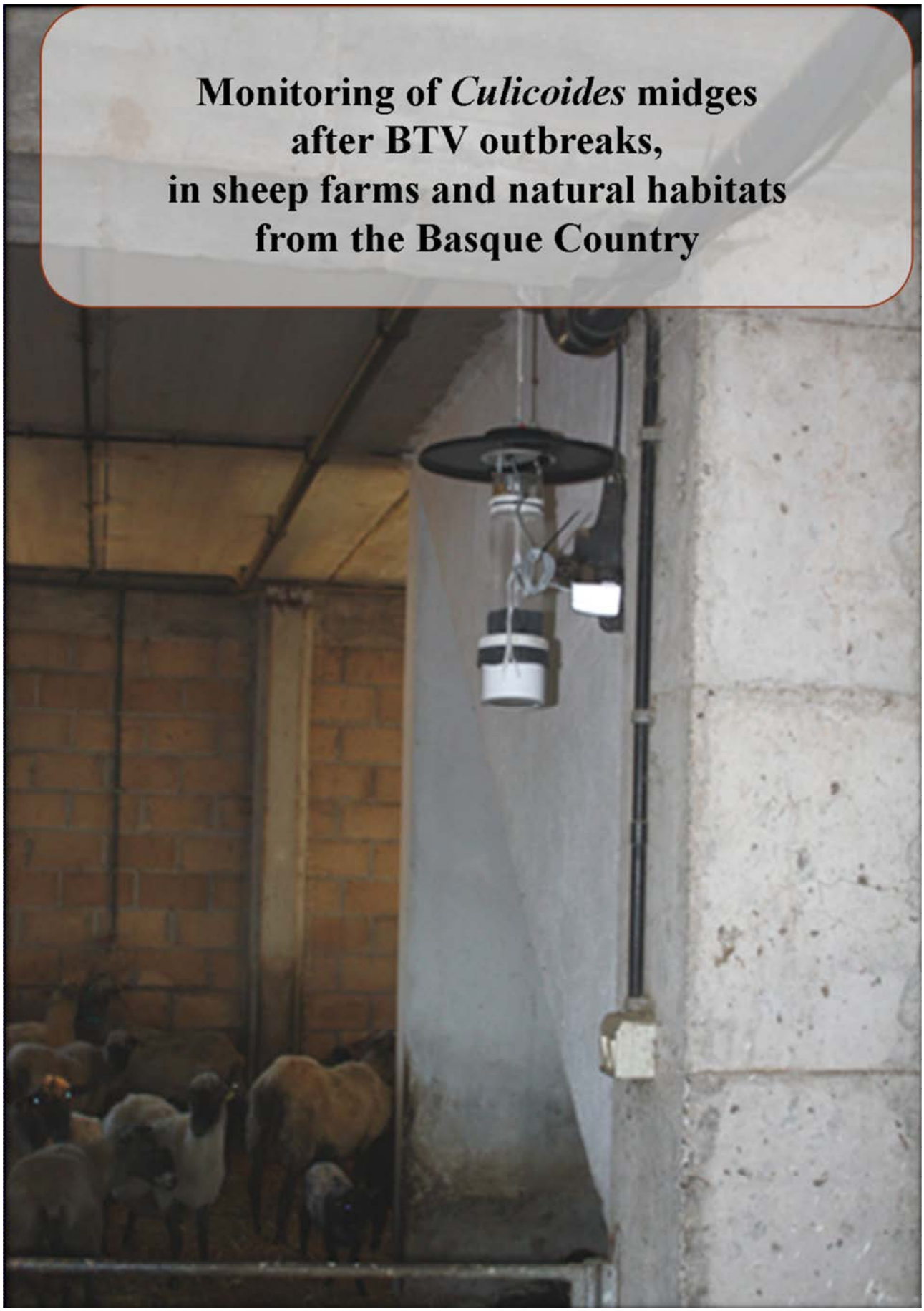
CHAPTER 2

Version of the chapter 2 based on the following publication:

Monitoring of *Culicoides* Latreille (Diptera: Ceratopogonidae) after BTV outbreaks, in sheep farms and natural habitats from the Basque Country (Northern Spain). 2013. Proceedings of the Entomological Society of Washington, 115 (1): 48-69.

Mikel González, Thierry Baldet, Jean Claude Delécolle, Sergio López, Pedro Romón and Arturo Goldarazena


Monitoring of *Culicoides* midges
after BTV outbreaks,
in sheep farms and natural habitats
from the Basque Country

MONITORING OF *CULICOIDES* MIDGES AFTER BTV OUTBREAKS, IN SHEEP FARMS AND NATURAL HABITATS FROM THE BASQUE COUNTRY

1. ABSTRACT

Since bluetongue outbreaks occurred in November 2007 in northern Spain, an intensive trapping program was carried out to study the diversity and abundance of the *Culicoides* (Diptera: Ceratopogonidae) species. Eleven sheep farms and seven natural habitats distributed throughout the Basque Country region were sampled using UV light CDC traps between 2008-2011. A total of 348,685 *Culicoides* specimens belonging to 52 species were collected during 1,480 trappings in 24h periods. An updated checklist of these 52 species (including four new records for the Iberian Peninsula) is provided from the Basque country. The most abundant species in sheep farms were the two sibling species of the Obsoletus complex (81.8% of the total catches): *C. obsoletus* and *C. scoticus*. *Culicoides lupicaris* was the next most abundant taxon collected in farms (9.7% of the total). Few specimens of *Culicoides imicola*, the Bluetongue vector in Mediterranean Basin and some specimens of *Culicoides nubeculosus*, an important candidate of Bluetongue virus transmission were collected in northeast and northwest farms, respectively. In natural ecosystems, three species, *Culicoides festivipennis*, *Culicoides alazanicus* and *Culicoides brunnicans*, comprised 48.5% of the total captures. *Culicoides obsoletus/C. scoticus* was present throughout the year and even during the winter days in temperate Atlantic areas (Gipuzkoa, Biscay) whereas no catches occurred in winter at the southern farms of Alava, where the climate is much colder in that season. The majority of the species were active from March to November with maximum peaks of catches in summer. Most *Culicoides obsoletus/C. scoticus* specimens were collected outdoors (65.9%). The study shows greater occurrence and abundance of Obsoletus and Pulicaris complex in sheep farms and in lesser extent in natural ecosystems. Data about diversity, distribution and seasonal dynamics are also reported improving the knowledge of these *Culicoides* species in terms of surveillance and prevention for future bluetongue outbreaks.

2. KEY WORDS Obsoletus complex, Pulicaris complex, Diversity, Distribution, Seasonal dynamics, Species richness, Wild/livestock environments, Light traps

3. INTRODUCTION

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are small hematophagous dipterans with 1,322 extant species (Borkent, 2012) distributed worldwide ranging from tropics to the tundra and from sea level to 4,000 m with the exception of New Zealand and Antarctica (Mellor et al. 2000). The genus is especially important because of the blood-sucking habits of females of most *Culicoides* species and the transmission of two main diseases to livestock with serious veterinary impact and economical losses: Bluetongue virus (BTV) and African horse sickness (AHS), with as many as 30 species of *Culicoides* worldwide hypothesized to be capable of transmission (Meiswinkel et al. 2004).

Transmission of BTV in Spain is associated with several species of *Culicoides*, all belonging to the subgenus *Avaritia*, with *C. imicola* the main vector of bluetongue virus in Spain (Acevedo et al. 2010) and the most important candidate and responsible for at least 90% of BTV transmission in the Mediterranean Basin (Meiswinkel et al. 2008a). However, two sibling species of the Obsoletus complex *C. obsoletus* and *C. scoticus* have been implicated in BTV transmission in the field in Italy (De Liberato et al. 2005; Savini et al. 2005), Bulgaria (Purse et al. 2006a) and Germany (Hoffmann et al. 2009) and in the laboratory (Carpenter et al. 2006, 2008). In the Netherlands, BTV-8 has been detected in others members of the Obsoletus group, i.e., *C. chiopterus* and *C. dewulfi* (Meiswinkel et al. 2007; Dijkstra et al. 2008). Two species of the Pulicaris complex, *C. pulicaris* and *C. lupicaris*, also are important candidates for BTV transmission (Mellor et al. 1990; Caracappa et al. 2003; Romón et al. 2012). *Culicoides nubeculosus* might serve as a vector, because it occurs abundantly near livestock and feeds on mammals. Experimental infections have shown the virus can replicate in *C. nubeculosus* at high temperatures, indicating this species could transmit BTV during climatically warmer periods (Wittmann, 2000). Moreover, it belongs to the same subgenus as *C. sonorensis*, the most important vector of BTV in the United States (Sarto i Monteys et al. 2009).

In Europe, bluetongue was reported for the first time in 1924 in Cyprus. Between 1956 and 1960, BTV-10 emerged in Southern and Central Spain causing the death of more than 179,000 sheep (Ortega et al. 1999). The affected regions coincided with the distribution area of *C. imicola*, considered the main BTV vector in Africa and the Middle East. *Culicoides imicola* was first reported in the European continent in southern Iberia (Mellor et

al. 1983; Mellor et al. 1985) and this Afro-asiatic species had not been recorded elsewhere in Europe despite entomological investigations in the 1970s (Mellor et al. 2000).

Between 1998 and 2005, BTV outbreaks occurred in many countries around the Mediterranean basin (Mellor and Wittmann, 2002), including BTV-4 outbreaks in Balearic Islands from 2000-2003 and in Andalusia in 2004. This transmission was associated with the northward extension of *C. imicola*, which colonized northern Mediterranean territories such as the Balearics Islands during the past decades probably due to the global warming (Purse et al. 2006b; Guis et al. 2012). As a consequence, a national surveillance program was initiated across Spain to estimate the spatial and seasonal distribution of *Culicoides* species associated with livestock (Calvete et al. 2009).

In August 2006, BTV-8 was introduced in The Netherlands and was intensively transmitted by autochthonous Palaearctic *Culicoides* species (Meiswinkel et al. 2008a; Saegerman et al. 2008), leading to a major sanitary crisis in 2007 and 2008 in central Europe.

In November of 2007, BTV-1 reached the Basque Country, Navarre and Atlantic Pyrenean areas. A total of 546 outbreaks (61 in 2007, 478 in 2008 and 7 in 2009) occurred in the Basque Country between 2007-2009, distributed as follows: 324 in cattle herds, 142 in sheep flocks, 6 in goat flocks and 74 in mixed farms. No more outbreaks have been notified since March of 2009. In contrast, several occasional outbreaks have been reported in other provinces of Spain during this period. A total of 100 outbreaks have been notified in the last three years compared to more than 11,890 occurred in 2004-2009 (RASVE, 2012).

Due to outbreaks of BTV in northern part of the Iberian Peninsula, two parallel entomological surveillance programs were carried out, one in some localities of the Autonomous Community of Basque Country (Lucientes et al. 2008; MARM, 2008; Calvete et al. 2009) and the other focusing on Gipuzkoa province (Goldarazena et al. 2008; Romón et al. 2012). Accounts of species composition were provided by Goldarazena et al. (2008), reporting the first catches of *C. imicola* in some stables of Gipuzkoa. No data about the fauna of *Culicoides* in Biscay and Alava provinces had been reported.

In the current study we present for first time the results of an annual sampling program in eleven sheep farms of the Basque Country region, including Biscay and Alava provinces, and a seasonal sampling for seven natural habitats, reporting the species richness,

abundance and seasonal dynamics of *Culicoides* both potential and non-potential vectors of BTV.

4. MATERIAL AND METHODS

4.1. Study area and period of sampling

Eleven sheep farms were chosen following four criteria: farms affected by bluetongue virus, minimum of 80 sheep, different bioclimatic areas and no use of insecticides. The region of study included the provinces of Gipuzkoa (3 farms), Biscay (3 farms) and Alava (5 farms) (Fig. 1, Table 1). Sampling was carried out every day for 24 hours in all sites during 12 months (Gipuzkoa in 2008, Alava in 2009 and Biscay in 2010). In addition, seven natural habitats (wetland, grassland, meadow-tree-patch ecotone, pine forest, oak grove, pond and Mediterranean shrubland) were surveyed for the presence of *Culicoides* (Fig. 1, Table 1). Traps were run from the first week of April to the last week of September 2011 for 48h once a week.

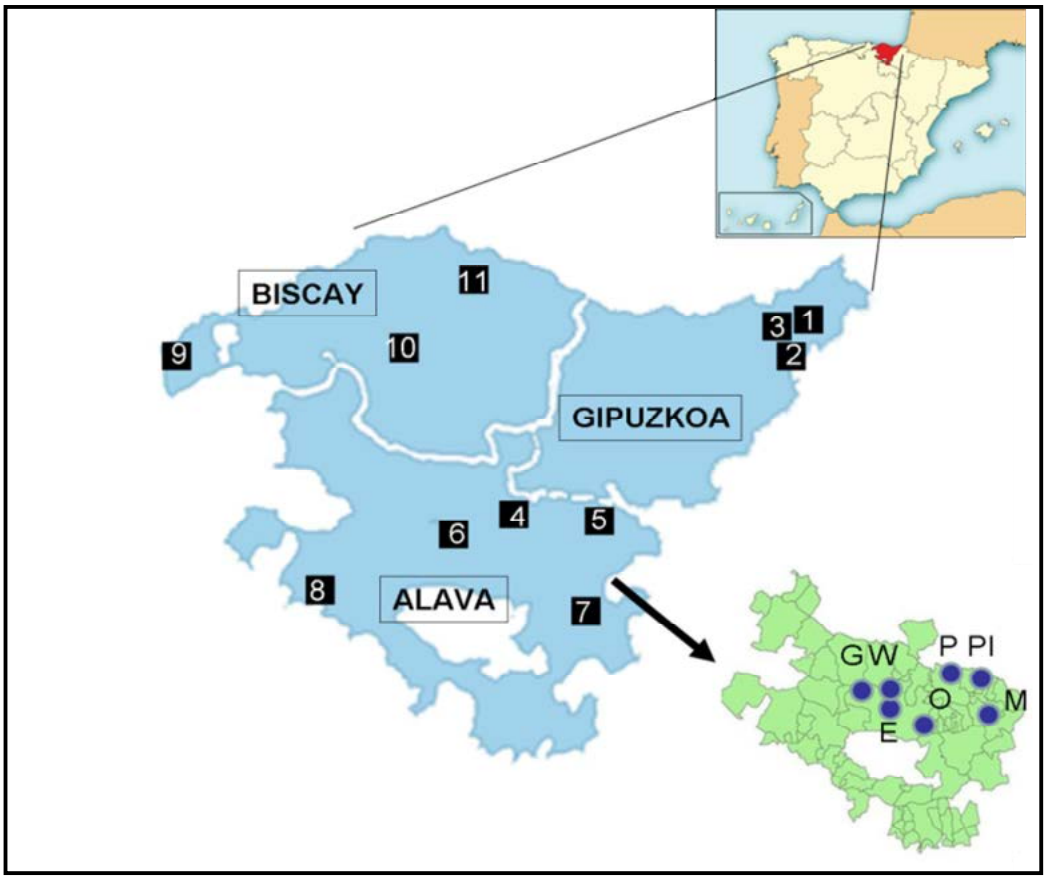


Fig. 1. Map of Basque Country (Northern Spain) showing the 18 sampling sites for *Culicoides*, 2008-2011. Sheep farms Gipuzkoa: **(1)** Azkue **(2)** Oiantzabal **(3)** Zabaltzo; Alava: **(4)** Elguea **(5)** Zalduondo **(6)** Arkaute **(7)** San Vicente de Arana **(8)** Zambrana; Biscay: **(9)** Carranza **(10)** Orobio **(11)** Markina; Natural habitats: **(W)** Wetland **(G)** Grassland **(O)** Oak grove **(M)** Mediterranean shrubland **(P)** Pond **(PI)** Pine forest **(E)** Ecotone.

Table 1. Location and data about the 18 sampling sites (2008-2011). Numbers and letters in brackets correspond to the sampling sites in Figure 1.

Sampling sites	Province	Geographic coordinates	Altitude (m)	Type of site	Number of traps	Catch periodicity	Livestock/wild fauna
Azkue (1)	Gipuzkoa	30T0594033	145	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2008	Sheep >200
Oiantzabal (2)	Gipuzkoa	30T0593489	80	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2008	Sheep 200 ~
Zabaltxo (3)	Gipuzkoa	30T0591409	110	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2008	Sheep >100
Elguea (4)	Alava	30T0549088	754	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2009	Sheep >300 and horses sometimes
Zalduondo (5)	Alava	30T0553634	608	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2009	Sheep >100
Arkaute (6)	Alava	30T0530122	514	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2009	Sheep 80 ~
San Vicente de Arana (7)	Alava	30T0552387	804	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2009	Sheep >100
Zambrana (8)	Alava	30T0510132	485	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2009	Sheep >100 and horses
Carranza (9)	Biscay	30T0483939	751	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2010	Sheep 150
Orobio (10)	Biscay	30T0527188	430	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2010	Sheep >150, 10 dogs
Markina (11)	Biscay	30T0540831	85	Sheep farm	2 (indoor + outdoor)	Weekly throughout 2010	Sheep >150
Salburua (W)	Alava	30T0528723	578	Wetland	1 (outdoor)	From April to November 2011	Aquatic birds (coots, ducks), Red deers
Salburua (G)	Alava	30T0529011	582	Grassland	1 (outdoor)	From April to November 2011	Birds, rodents and Red deers
Cerio (O)	Alava	30T0532646	590	Oak grove	1 (outdoor)	From April to November 2011	Ducks, wild boars, rodents
Etura (M)	Alava	30T0541722	670	Mediterranean shrubland	1 (outdoor)	From April to November 2011	Birds, cattle, rabbits
Barrundia (P)	Alava	30T0546942	733	Pond	1 (outdoor)	From April to November 2011	Ducks and ungulates
Barrundia (PI)	Alava	30T0544105	720	Pine forest	1 (outdoor)	From April to November 2011	Horses, rodents, birds
Arkaute (E)	Alava	30T0529979	540	Ecotone	1 (outdoor)	From April to November 2011	Ungulates and birds

4.2. Monitoring method

Midge collection from sheep farms was made with suction light traps (CDC blacklight UV 4 W tube model 1212, Entomopraxis, Barcelona, Spain) in non-stop night and day by using 200-12V transformers connected to the general power network of the farms. On each farm, two traps were set, one indoors and another outdoors, suspended at a height of 2.5 m from man-made structures and located within 10 m of sheep. Specimens of *Culicoides* were collected into a reusable plastic jar suspended below the fan of the trap, half-filled with 1,2-propanodiol (99% purity). Mosquito netting (size: 5 mm) was used to prevent larger insects from entering light trap. Each Tuesday, jars were collected and traps were checked in order to keep the equipment in working order.

Sampling method for the natural habitats consisted of modified light traps (CDC 6 V portable traps with incandescent light removed and replaced with a 390 nm ultraviolet light Led array, Bioquip Products, Rancho Dominguez, U.S.A) due to the higher abundance and diversity of UV light over white incandescent light for the collection of *Culicoides* midges (Venter and Hermanides, 2006; Venter et al. 2009). These traps worked using 4 D-cell batteries fitted into a plastic holder that supplied power for 48h-period operations. Traps were hung on a tree branch (2-2.5 m above the ground), having previously eliminated the

ground level vegetation from the area. The beaker was filled with water to which a few drops of detergent were added. Beakers were removed and batteries replaced weekly.

4.3. Identification

Specimens of *Culicoides* were initially sorted according to wing pattern, using a Leica MZ95 stereoscopic microscope and taxonomic keys (Delécolle, 1985; Glukhova, 2005), and fixed in 90% ethanol. Specimens that were difficult to classify were mounted on glass slides in Hoyer medium (50 ml of distilled water, 30 g gum arabic, 200 g chloral hydrate, 20 ml glycerin) and subsequently identified with a Leica DM4500B microscope, using various additional morphological characters. For species complexes (e.g. *C. obsoletus/C. scoticus*) that females cannot be separated morphologically, the species were recorded as a species complex (e.g. *C. obsoletus*/*C. scoticus*).

All the collected specimens were identified, counted and separated by sex. The microscope slides consisted on *Culicoides* dissected in four parts: head (dorsal), abdomen (ventral), thorax (dorsal) and legs (lateral). Voucher specimens were deposited at the Entomology Collection of the NEIKER-Basque Institute for Agricultural Research and Development, Basque Country, Spain.

5. RESULTS AND DISCUSSION

Up to 1,480 collections were performed in 24h periods: a total of 348,685 trapped specimens of 52 species of *Culicoides* were morphologically identified (330,783 females, 14,982 males).

On sheep farms, 345,768 midges of 48 species were collected: 130,451 specimens of 30 species in Gipuzkoa, 165,434 specimens of 43 species in Alava and 49,883 specimens of 30 species in Biscay (see Table 3-5 at the end of this chapter) during 1,144 non-stop collections. In natural habitats, 2,917 specimens of 31 species were collected over 336 days. In the ecotone and oak grove, 600-700 specimens were collected, grassland, pond and pine forest 400-500 specimens and wetland and Mediterranean shrubland with 90-155 specimens (Fig. 2).

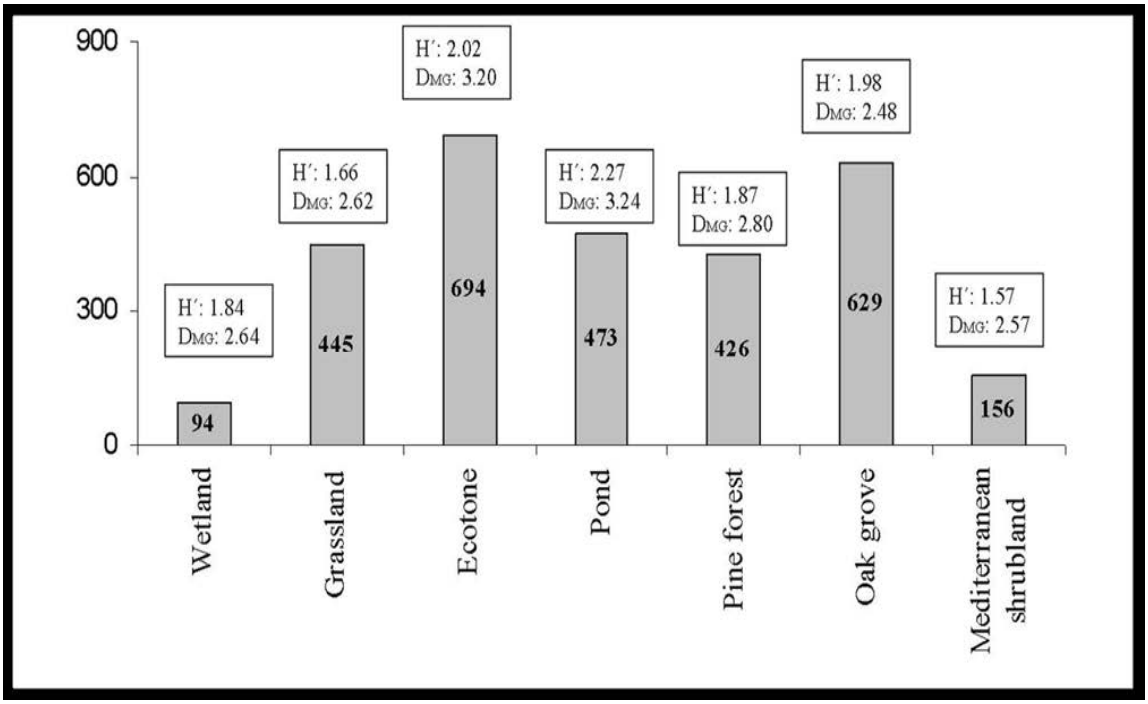


Fig. 2. Total *Culicoides* catches for each ecosystem in natural habitats from April to November 2011 with portable CDC-UV traps. Shannon-Weiner Index (H´) and Margalef Diversity Index (D_{MG}) are provided.

5.1. Faunistic inventory

We reported 52 species of which four were new records to the Iberian Peninsula: *C. heliophilus, C. paradisionensis, C. clastrieri* and *C. tauricus.* An updated list of *Culicoides* in the Basque Country territory and data about their presence/absence in the different sampling sites are provided in Table 2.

In regards to Spanish mainland diversity, the contributions mainly carried out by De Prada and Gil Collado (1959), Sahuquillo Herráiz and Gil Collado (1982), Mellor et al. (1983), Rawlings (1996) and Delécolle (2002) provided detailed information about the 61 reported species from Spain, Portugal and Andorra. Subsequently, during the following 10 years the number of species has been increased to 81 *Culicoides* species from Spain, including our four new recordings. Ramilo et al. (2012) have recently reported the *Culicoides* species distribution in mainland Portugal. Actually, *Culicoides* species in Spain is divided in 9 subgenera, a miscellaneous group and a *nomen dubium* (Alarcón-Elbal and Lucientes, 2012).

Table 2. Updated list of *Culicoides* species recorded in the Basque Country (Spain) and presence or absence in the sampling sites. (*) New records to the Iberian Peninsula. Sheep farms **(1)** Azkue **(2)** Oiantzabal **(3)** Zabaltzo **(4)** Elguea **(5)** Zalduondo **(6)** Arkaute **(7)** San Vicente de Arana **(8)** Zambrana **(9)** Carranza **(10)** Orobio **(11)** Markina; Natural habitats: **(W)** Wetland **(G)** Grassland **(O)** Oak grove **(M)** Mediterranean shrubland **(P)** Pond **(PI)** Pine forest **(E)** Ecotone.

Culicoides species	Distribution on sheep farms				Distribution on natural habitats	
	Gipuzkoa	Alava	Biscay	Σ		Σ
1.*C. achrayi* Kettle and Lawson, 1955	1,2,3	4,5,7	9,10,11	9	O, P,PI	3
2.*C. alazanicus* Dzhafarov, 1961	–	4,6,7	–	2	W,G,O,M,PI,E	6
3.*C. brunnicans* Edwards, 1939	–	4,5,6,7	9,10,11	7	G,O,M,P,E	5
4.*C. cataneii* Clastrier**,** 1957	1,2	5,8	9,10	5	–	–
5.*C. chiopterus* (Meigen, 1830)	1,2,3	4,5	9,10,11	8	–	–
6.*C. circumscriptus* Kieffer, 1918	1,3	4,5,6,7,8	9,10,11	10	E	1
7.*C. clastrieri* Callot, Kremer and Deduit, 1962 *	–	4	–	1	W	1
8.*C. comosioculatus* Tokunaga, 1956	1	–	–	1	–	–
9.*C. dewulfi* Goethebuer, 1936	1,2	4,5,7,8	9,10	8	G,M,E,P	4
10.*C. duddingstoni* Kettle and Lawson, 1955	–	–	–	–	W,G,O,M,E	5
11.*C. fagineus* Edwards, 1939	1,2,3	4,5,7	10	7	E	1
12.*C. fascipennis* (Staeger, 1839)	1,2	4,5,7,8	–	6	P,PI	2
13.*C. festivipennis* Kieffer, 1914	1,2,3	4,5,6,7,8	9,10,11	11	W,G,O,P,PI,E	6
14.*C. flavipulicaris* Dhafarov, 1964	2	–	–	1	–	–
15.*C. furcillatus* Callot, Kremer and Paradis, 1962	1,2	4,5	9,10,11	7	O,PI,E	3
16.*C. gejgelensis* Dzhafarov, 1964	–	4,5,6,8	9,10,11	7	G,E	2
17.*C. griseidorsum* Kieffer, 1818	–	4,6,8	–	3	W,G,E	3
18.*C. heliophilus* Edwards, 1921 *	–	4	–	1	PI	1
19.*C. imicola* Kieffer, 1913	2,3	–	10	3	–	–
20.*C. impunctatus* Goetghebuer, 1920	1	5	10	3	–	–
21.*C. odiatus* Khalaf, 1961	1	4,5,7	10	5	–	–
22.*C. kibunensis* Tokunaga, 1937	1,2	4,5,6,7,8	9,10,11	10	W,G,O,M,P,PI,E	7
23.*C. kurensis* Dzhafarov, 1960	–	4	–	1	–	–
24.*C. longipennis* Khalaf, 1957	–	4	–	1	PI	1
25.*C. lupicaris* Downes & Kettle, 1952	1,2,3	4,5,6,7,8	9,10,11	11	G,O,M,P,PI,E	6
26.*C. maritimus* Kieffer 1924	–	–	10,11	2	G,E	2
27.*C. minutissimus* (Zettertedt, 1855)	1	8	10	3	–	–
28.*C. newsteadi* Austen, 1921	1,2,3	4,5,6,7,8	9,10	10	G,P,E,O	4
29.*C. nubeculosus* (Meigen, 1830)	1	5	9	3	–	–
30.*C. obsoletus* (Meigen, 1818)	1,2,3	4,5,6,7,8	9,10,11	11	W,G,O,M,P,PI,E	7
31.*C. pallidicornis* Kieffer, 1919	1,2	4,5	9,10,11	7	P	1
32.*C. paradisionensis* Boorman, 1988 *	–	4	–	1	–	–
33.*C. parroti* Kieffer, 1922	1	4,6	9	4	–	–
34.*C. pictipennis* Staeger, 1839	–	4,6	10	3	W,G,O,P,PI,E	6
35.*C. picturatus* Kremer and Deduit, 1961	–	4,5,7,8	9	5	P,PI	2
36.*C. poperinghensis* Goethebuer, 1953	–	4,5,6,7	11	5	W,G,O,M,P,PI,E	7
37.*C. pseudoheliophilus* Callot & Kremer, 1961	–	–	–	–	O,M,P,PI	4
38.*C. pulicaris* (Linnaeus, 1758)	1,2,3	4,5,6,7,8	9,10,11	11	W,P	2
39.*C. punctatus* (Meigen, 1804)	1,2,3	4,5,6,7,8	9,10,11	11	W,G,O,M,P,PI,E	7
40.*C. riethi* Kieffer, 1914	1	–	–	1	–	–
41.*C. santonicus* Callot, Kremer, Rault and Bach, 1966	–	4,5	–	2	–	–
42.*C. scoticus* Downes & Kettle, 1952	1,2,3	4,5,6,7,8	9,10,11	11	W,G,O,M,P,PI,E	7
43.*C. semimaculatus* Clastrier, 1958	3	5	–	2	–	–
44.*C. shaklawensis* Khalaf, 1957	–	4	–	1	P	1
45.*C. simulator* Edwards, 1939	–	4,5,7	–	3	P,E,PI	3
46.*C. stigma* (Meigen, 1818)	1,2	4,6	9,11	5	–	–
47.*C. subfagineus* Delécolle and Ortega, 1998	1	–	–	1	–	–

Table 2. Continued.

48.*C. subfasciipennis* Kieffer, 1919	1,2	5,7	–	5	–	–
49.*C. tauricus* Gutsevich, 1959 *	–	4,5,7,8	–	4	–	–
50.*C. truncorum* Edwards, 1939	–	–	–	–	M	1
51.*C. univitattus* Vimmer, 1932	–	4,5,6,7,8	10	6	W,G,O,M,P,PI,E	7
52.*C. vexans* Staeger, 1839	–	4,5,6,7,8	10	6	O,M,P,E	4
Σ	27,20,13	37,31,19,23,18	21,25,17	11	13,17,17,14,21,18,21	7

The total number of *Culicoides* species present in the Iberian Peninsula is difficult to predict accurately. The reason might be (i) *Culicoides* collections are scarce or lacking in many areas, (ii) the persistence of synonyms and taxonomic disagreements and (iii) not all species are attracted to light and thus might be missed by light trapping. Future studies combining both molecular and morphological approaches and other collection methods may provide a more complete list of the Iberian fauna.

Two principal bioclimatic regions are present in the Basque Country; Gipuzkoa and Biscay where oceanic climate is predominant with wet weather year round and moderate temperatures and Alava with continental Mediterranean climate, in which summers are dry and warm and winters cold and snowy. Therefore, species like *Culicoides brunnicans, Culicoides vexans, Culicoides furcillatus, Culicoides achrayi, Culicoides univitattus, Culicoides simulator* and *Culicoides tauricus* were present in greater numbers in many farms of Alava whereas they were absent or in low number in northern farms of Biscay and Gipuzkoa. Similar differences in diversity of *Culicoides* species depending on climatic factors are found by Talavera et al. (2011) in northeast Spain.

5.2. Species distribution

The current species distribution varied between the two groups of sampling sites: sheep farms and natural habitats. In both groups, *Culicoides* midges were present in high numbers, but with different composition of abundance.

The most common *Culicoides* species at the livestock farms were *C. obsoletus/C. scoticus*, a grand total of 282,710 specimens (81.8%) followed by *C. lupicaris* 33,382 (9.7%). The remaining 8.5% belonged to *C. punctatus* 7,082 (2.1%), *C. furcillatus* 6,842 (2.0%), *C. achrayi* 3,129 (0.9%) and other scarcer species (Tables 3-5). *Culicoides chiopterus* and *C. dewulfi* were collected in much lower quantities, with 0.10% and 0.02% of the total catches, respectively. The implementation of the trappings in sheep farms instead

on cattle farms, could explain the scarcity of the these both species in our results (Zimmer et al. 2008; Ninio et al. 2010).

The respective proportion of *C. obsoletus*/*C. scoticus* was estimated by identification of males, which revealed 91.5% belonged to *C. obsoletus* and 8.5% to *C. scoticus*, whereas 400 females randomly selected from different farm collections, mounted on slides and identified morphologically (Augot et al. 2010; Nielsen and Kristesen, 2011) indicated 85% belonged to *C. obsoletus* and 15% to *C. scoticus*. The *C. obsoletus*/*C. scoticus* ratio obtained in sheep farms distributed throughout the Basque country was similar to ratios found by Gerry et al. (2009) in Catalonia (northeast Spain) and Foxi et al. (2011) in Sardinia. The PCR results via multiplex obtained in Portugal by Ramilio et al. (2012) were slightly different: 69.6% *C. obsoletus*; 30.4% *C. scoticus*. Nevertheless, it is worthwhile to highlight that this prevalence varies among countries, being *C. scoticus* catches higher than those of *C. obsoletus* in farms of France (Baldet et al. 2004; Cêtre-Sossah et al. 2004) and southwest Germany (Balczun et al. 2009).

Other interesting potential vectors of BTV were collected. In November and December of 2007, Goldarazena et al. (2008) captured for first time six specimens of *C. imicola* in two localities of Gipuzkoa. The currently intensive monitoring program has allowed trapping in Gipuzkoa two new specimens of *C. imicola* in July and August of 2008. No specimens were captured in Alava, but one was collected indoors in Orobio (Biscay) in winter 2010. Two main hypotheses have been postulated to explain the arrival of this Afro-asiatic species at these northern latitudes. The first is the entrance of livestock incorrectly fumigated from areas of Spain where *C. imicola* is native. The second is passive dispersal of midges by flowing of warm air masses from southern areas of the Iberian Peninsula where large populations of *C. imicola* occur (Mellor et al. 2000; Goldarazena et al. 2008; Sanders et al. 2011a; García-Lastra, 2012). Passive dispersal by wind could explain the recent settlement of *C. imicola* in Catalonia (northeast Spain) from Balearics Island, some 200 km south-east offshore where it has been found in high abundance (Sarto i Monteys et al. 2005).

At the time, this finding in Catalonia was also the northernmost point where *C. imicola* had been recorded on the eastern side of the Iberian Peninsula (41°35´N), matching that of Salsas (Portugal) on the western side. The nearest point with important established populations of *C. imicola* in the southern Spain is in Ávila (Castille) about 350 km far away from the Basque Country frontier and in the eastern, considerable collections have been

reported in Zaragoza, at a distance of 200 km (Lucientes et al. 2008). Further studies are required to determine whether *C. imicola* populations will become well established in northern Spain. Some specimens of *C. nubeculosus* another potential BTV vector have also been collected in the Basque country: three in Gipuzkoa, one in Alava and 101 in Biscay.

In natural samplings sites, the dominant species were *C. festivipennis* with 486 specimens (19.3%), *C. alazanicus* with 448 (17.7%) and *C. brunnicans* with 291 (11.5%). These three species comprised 48.5% of the midges collected (Fig. 3); the remaining fauna belonged to 28 other species.

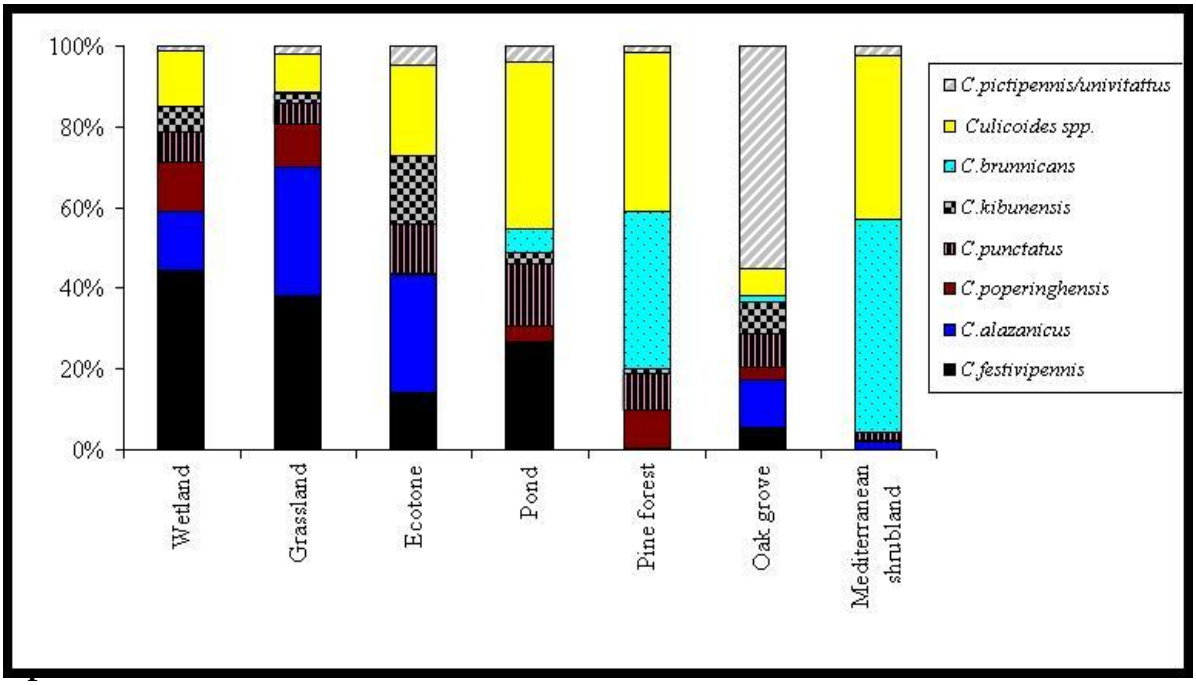


Fig. 3. Relative composition of the most abundant *Culicoides* species collected by UV-light CDC traps in seven natural habitats of the Basque country from April to November 2011. *Culicoides* spp. includes 23 scarcer species.

Species composition between farm settings and natural habitats were markedly different. *Culicoides obsoletus/C. scoticus* complex was clearly predominant in the sheep farms, in contrast to the natural sampling habitats, where *Culicoides* catches varied between 0.5-10% as compared to the 70-85% in farms. In addition, *C. scoticus* was more abundant, accounting 25% of the total specimens of *C. obsoletus/C. scoticus* collected (based on male diagnosis). In addition, the widespread presence of *C. obsoletus*/*C. scoticus* in farm surrounding environments could have a strong association with livestock availability and with their main larvae developing sites, associated with different types of manure substrates (González et al. 2013). The abundance of the second common taxon (*C. lupicaris*) in farm settings was minority in natural environments with less than 35 captured specimens (distributed mainly at the pond and in the pine forest). However, Pulicaris group was found

in a great variety of farm environments in high numbers, particularly in areas near water (González et al. 2013) and for that it is likely that their presence is markedly conditioned not only by the host preferences (mainly livestock) but also by other factors like: (i) type of habitat (ii) soil characteristics (pH, water fluctuation, oxygen availability, percentage of organic matter, predators, chemical composition of minor constituents) and (iiii) climate factors (temperature, air humidity, light intensity, wind speed) could be important determinants in the habitat occupation.

However, the high abundance of *C. festivipennis* mainly in wetland, grassland, and pond natural habitats could be attributed to the affinity of this species to breed on wet organic environments, particularly substrates near pools of water, mud rich in organic matter and inundated soils near water line (Foxi and Delrio, 2010; Uslu and Dik, 2010; González et al. 2013). Likewise, *C. festivipennis* seems to be a ornithophilic species, which are abundant in those places with wild aquatic birds (Foxi and Delrio, 2010; Lassen et al. 2012) but also might be host generalist (Calvo et al. 2012; Santiago-Alarcón et al. 2012). Most likely *C. alazanicus* (the second dominant taxon) appears to be an avian feeder, even so further studies of the abdomen blood-meal molecular analyses are necessary. In terms of species richness, the most species rich natural habitat was the pond and ecotone (Table 2). Moreover, natural habitats comprise three *Culicoides* species non encountered on farm settings: *C. duddingstoni*, *C. truncorum* and *C. pseudoheliophilus*.

The Obsoletus complex is the most common group of species found on sheep and cattle holdings across the entire Western Palearctic region. In Spain, Obsoletus complex are well spread through all the Iberian Peninsula, being abundantly captured in northern areas (Sarto i Monteys and Saiz-Ardanaz, 2003; Sarto i Monteys et al. 2009; Goldarazena et al. 2008; Romón et al. 2012; González and Goldarazena, 2011) while in southern Spain, *C. imicola* is the commonest species following by *C. obsoletus* complex and *C. newsteadi, C. pulicaris, C. nubeculosus* and *C. circumscriptus* are usually abundant depending on localities and dates of sampling (Ortega et al. 1998, 1999; Calvete et al. 2010; Pérez et al. 2012).

In our study, the most widespread species at sheep farms were *C. obsoletus*/*C. scoticus,* which appeared in all the sampled sites, whereas *C. dewulfi* and *C. chiopterus* were absent in some localities. Pulicaris group species including mainly *C. lupicaris* and to a lesser extent *C. pulicaris, C. punctatus* and *C. newsteadi* were also widely distributed,

missing only in one location (Markina in Biscay). Eleven of the 52 collected species were restricted to one farm, assuming a restricted distribution and only five species were present in all the farm sites: *C. festivipennis, C. lupicaris, C. obsoletus, C. scoticus* and *C. punctatus*.

On the other hand, *C. poperinghensis, C. punctatus, C. kibunensis* and *C. obsoletus/C. scoticus* were present in all the natural habitats whereas *C. alazanicus, C. festivipennis, C. pictipennis,* and *C. lupicaris* were captured in almost all habitats. Eight species were collected in one ecosystem being singletons or doubletons.

According to these data, the wide distribution and high abundance of the Obsoletus complex and the secondary abundance of the Pulicaris complex in livestock farms of the Basque country confirmed the entomological results reported in other countries of northern Europe (Meiswinkel et al. 2008a; Mehlhorn et al. 2009; Nielsen et al. 2010).

In contrast to most studies of *Culicoides* abundance which tend to concentrate on farms/stables with sheep, cattle, goats, horses or mix of them, the present study comprises a wide variety of habitats. *Culicoides* was strongly linked to land use and host density, with those areas surrounded by greater density of livestock returning greater numbers. Therefore, when comparing farm settings with natural settings in term of mean of *Culicoides* catches per day (considering that the sampling effort is different among settings, every day in farms, two days per week in natural habitats) in general seems to be observed more abundance of *Culicoides* collections in the first ones, suggesting that the presence of permanently livestock near farming encourage a high presence of *Culicoides* in those places according to Sanders et al. (2011b) in United Kingdom, where the total abundance of *Culicoides* increased significantly with the density of cattle in the locality of the trap and also mentioned that pastoral areas comprises higher abundances than mixed or arable habitat types.

5.3. Seasonal dynamics

Seasonal dynamics of *Culicoides* are driven by climatic factors, mainly rainfall and temperature. In the present study, *Culicoides* were recorded from April to November in farms of Alava but few midges were captured during the rest of the year; however, no specimens were collected in the coldest months in winter, reporting a vector free period in

January and February (Tª mean max. 9º C; Tª mean min. 0.4º C). Although *Culicoides* midges were active during the whole year in Gipuzkoa and Biscay, collections were markedly greater from March to November. In both latter provinces, only *Culicoides obsoletus/C. scoticus* and few specimens of the Pulicaris group were collected in the months of December, January and February. Sporadic captures of potential BTV vectors in the winter invite re-examination of the current definition of a vector-free period. Low temperatures at farms of the southern Basque Country regions (Alava) likely do not allow the survival of adult midges; a residual adult population might be able to hibernate at adequate sites or the population might overwinter as immatures, pupae or eggs (Wilson et al. 2008).

The period of maximum peaks varied among farms. April, June and July were the peak period of catches for Gipuzkoa, July for Alava and August for Biscay. These variations could be explained by the fact that collections were conducted in different years for each province. Generally, summer and beginning of autumn could be considered as the seasons with abundant populations and therefore a greater likelihood of transmission of *Culicoides*-borne diseases. *Culicoides* catches in Gipuzkoa increased in March and decreased from the last fortnight of July. Similar results were observed in Biscay but in April and November were observed important population peaks of *C. obsoletus/C. scoticus* and *C. lupicaris/C. pulicaris*), a little less notorious in Gipuzkoa. In Alava, the seasonal dynamics exhibited a greater increase in July that decreased sharply in August, a secondary peak was observed in November; collections of the rest of the year were scant except in May and July with relatively abundant captures.

Culicoides obsoletus/C. scoticus dominated all sampling months except October when species of the Pulicaris group (especially *C. lupicaris*) comprised 60% of the total catches (Fig. 4). Overall, *C. C. obsoletus/C. scoticus* displayed in our studied farms three season peaks: April, June-July and a reduced November peak. Although emergence pattern of this two species are less clear among authors, this pattern of seasonality for the Obsoletus complex was similar to that observed in Catalonia (Sarto i Monteys and Saiz-Ardanaz 2003) and in Balearic Islands (Miranda et al. 2004) but slightly different from the seasonal dynamics of *C. obsoletus/C. scoticus* reported in France, where these species were abundant around May and declined during summer dry months (Venail et al. 2012).

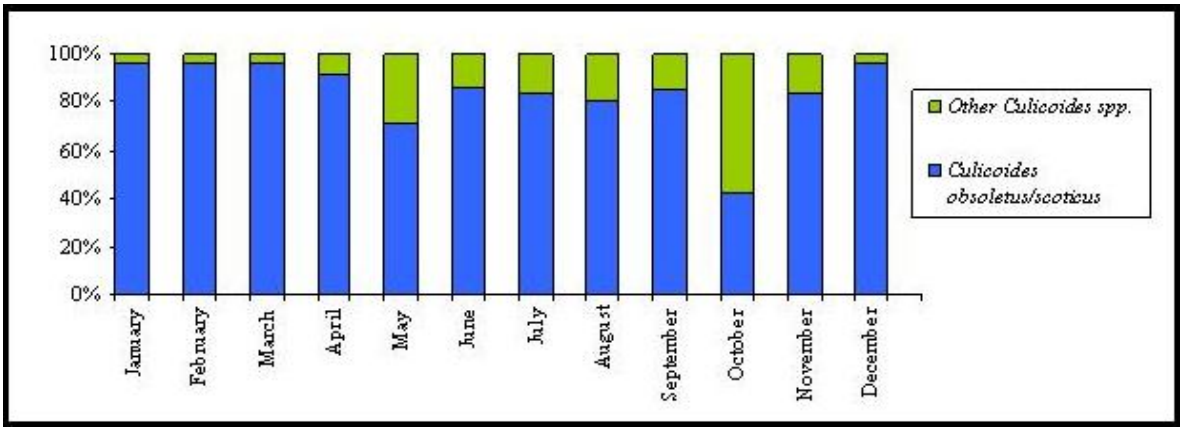


Fig. 4. Monthly variation of the relative composition of the Obsoletus complex and other *Culicoides* species collected by UV light CDC traps in 11 sheep farms of the Basque country during one year sampling.

Regarding the monthly variations of the relative composition within the Pulicaris group, *C. lupicaris* predominated all the months except in January when its abundance was similar to *C. pulicaris* (Fig. 5). In the sheep farms of the Basque country, *C. lupicaris* displayed two seasonal peaks (suggesting a bivoltine pattern): a larger one from April to July and a smaller one in October-November. These seasonal dynamics have also been observed for *C. pulicaris* in Sardinia (Foxi et al. 2011) and in United Kingdom (Sanders et al. 2011b). Our results are also according to Searle et al. (2012) which mentioned that the first generational peak (late April/early May) of *C. pulicaris* was on average larger that the second one (late summer/early autumn).

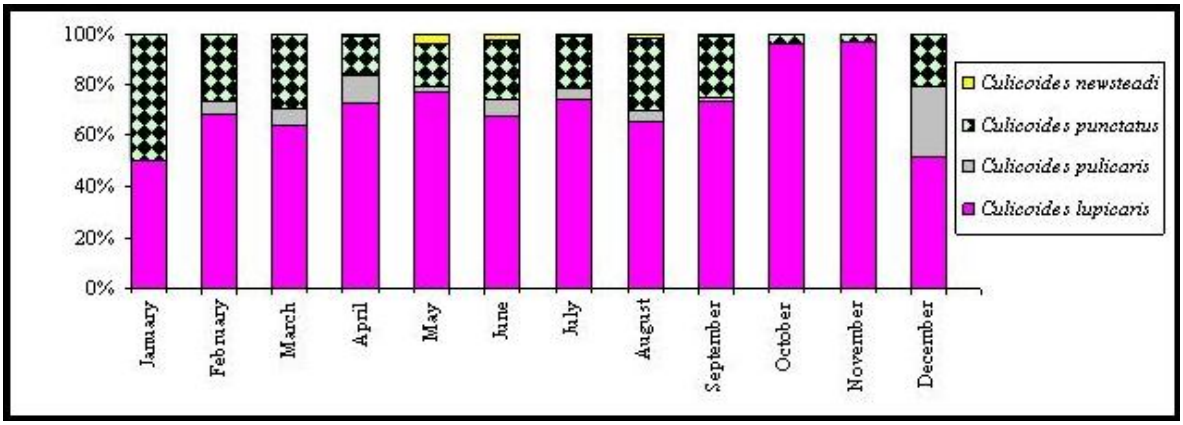


Fig. 5. Monthly variation of the relative composition of the Pulicaris group including: *C. lupicaris, C. pulicaris, C. punctatus,* and *C. newsteadi* collected by UV-light CDC traps in 11 sheep farms of the Basque country during one year sampling.

In natural ecosystems, May was the most abundant period of collections. *Culicoides* abundance decreased considerably in June, July and August and increased in September, before a raining period. In April only few *Culicoides* catches were recorded. May was the greatest month of *Culicoides* captures most likely because of the heavy rainfalls occurred during almost the whole April, which allowed the development of a great number of *C.*

brunnicans, C. punctatus and *C. pictipennis/C. univittatus*. As shown our CDC-farm collections, *C. brunnicans* seems to be very common at the end of the spring in grasslands.

5.4. Outdoors/indoors catches

Outdoor collections represented 65.9% of all *Culicoides* specimens. Species abundance was between 2-2.5 times greater outdoors than indoors, except in Biscay where these difference were less marked. Outdoors/indoors relative percentage catches of Obsoletus and Pulicaris complex are represented in the Fig. 6 and the seasonal dynamics of those species are shown in Fig. 7. The endo/exophagic biting and endo/exophilic resting behaviour of *Culicoides* species is a subject of debate. In Northern Europe, abundance of the Obsoletus group is generally lower inside farm buildings than outside (Nielsen et al. 2010) but several studies have shown that in late autumn and in winter when temperature falls and husbandry practices change (resulting in some animals being maintained permanently inside well-built cattle sheds till the following spring), a significant number of *C. obsoletus/C. scoticus* enter livestock buildings to feed (Baldet et al. 2008; Meiswinkel et al. 2008b; Zimmer et al. 2008). Venail et al. (2012) also pointed some important aspects such us the importance of animal presence/absence in indoor/outdoor collections. Indoor collections in the presence of animals were more abundant that outdoors and outdoor collections may be abundant even if animals were not present, but animal presence increased the number of collected *Culicoides* (mainly C. *obsoletus/C. scoticus*).

Baylis et al. (2010) reported some methodological mistakes in certain studies and therefore *C. obsoletus/C. scoticus* showed a degree of endophagic/endophilic tendencies, which varied from farm to farm depending on the season because of differential effects of weather conditions on outdoor catches. The persistence of significant numbers of members of the Obsoletus group towards the end of the season particularly inside holdings raises further their potential role in BTV overwintering. Viennet et al. (2012) highlighted that Palaearctic *Culicoides* species are primarily exophagous insects but *C. obsoletus/C. scoticus* showed some degree of endophagy in contrast to *C. brunnicans* strictly exophagous.

Moreover, light trap positions have a significant impact on the sampling results as demonstrated Lühken and Kiel (2012) in a study where observed that the number of females of *C. obsoletus* species group and to a lesser extent *C. pulicaris* species group significantly decreased with increasing distance to the stable. In addition they mentioned that trap

position (< 25m) from direct stables probably promises the highest trapping success. Nevertheless, it is thought that the size of animal housing was proportional with the entrance of *Culicoides* (Barnard, 1997) but in a recent study, Garcia-Saenz et al. (2010) observed that as the number of sheep increased from zero to three the number of midges caught increased, but there appeared to be no further increase when six sheep were used

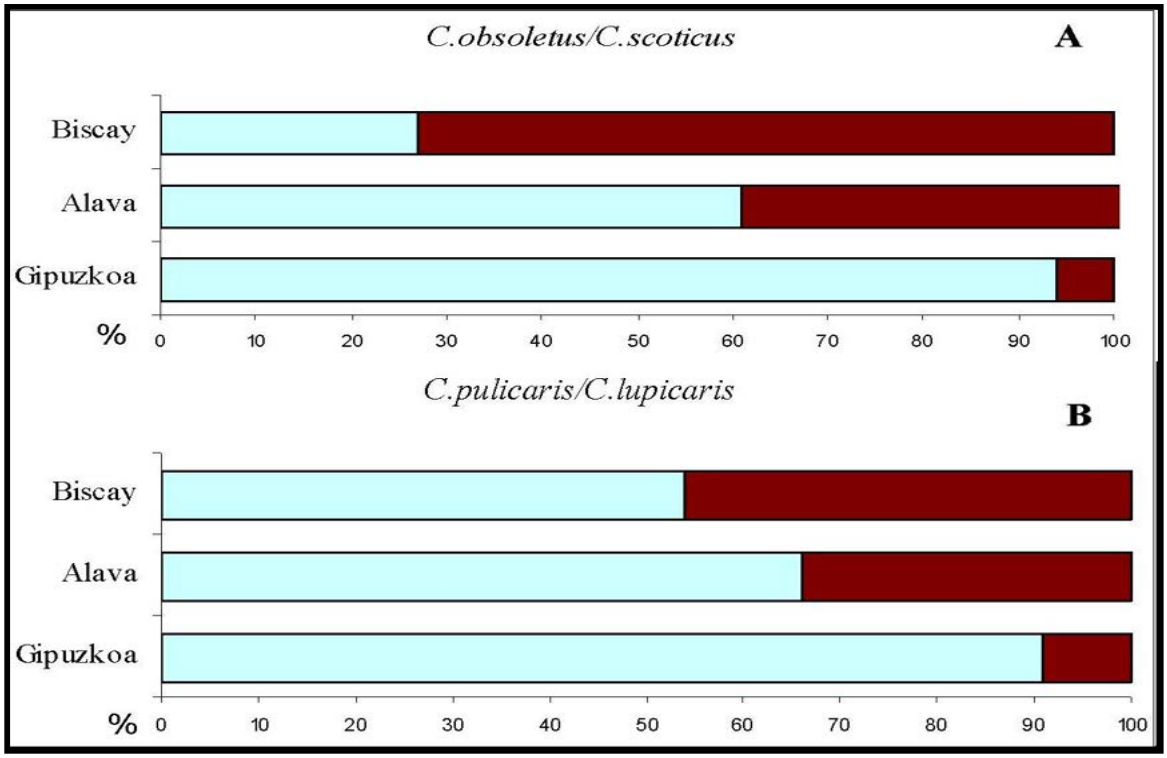


Fig. 6. Total percentage of *C. obsoletus/C. scoticus* **(A)** and *C. pulicaris/C. lupicaris* **(B)** catches. Outdoors (blue light bar) and indoors (red bar) in the three provinces of the Basque Country.

In contrast, members of the Pulicaris group, mainly *C. pulicaris*, exhibit a strong exophagic/exophilic behaviour in Europe (Meiswinkel et al. 2008a), with 15-20 times more abundant outside than inside holdings in the absence of animals (Baylis et al. 2010). However, livestock-management practices (i.e. animal densities in the near vicinity of the trap both in and outdoors) and the size of the entrances into sleeping sheds (opening or closing of the stable doors) are known as the most important factors that interfere in the entrance of *Culicoides* species (Barnard, 1997; Meiswinkel et al. 2008b; Baylis et al. 2010; Bell, 2011; M. González (unpbl.)).

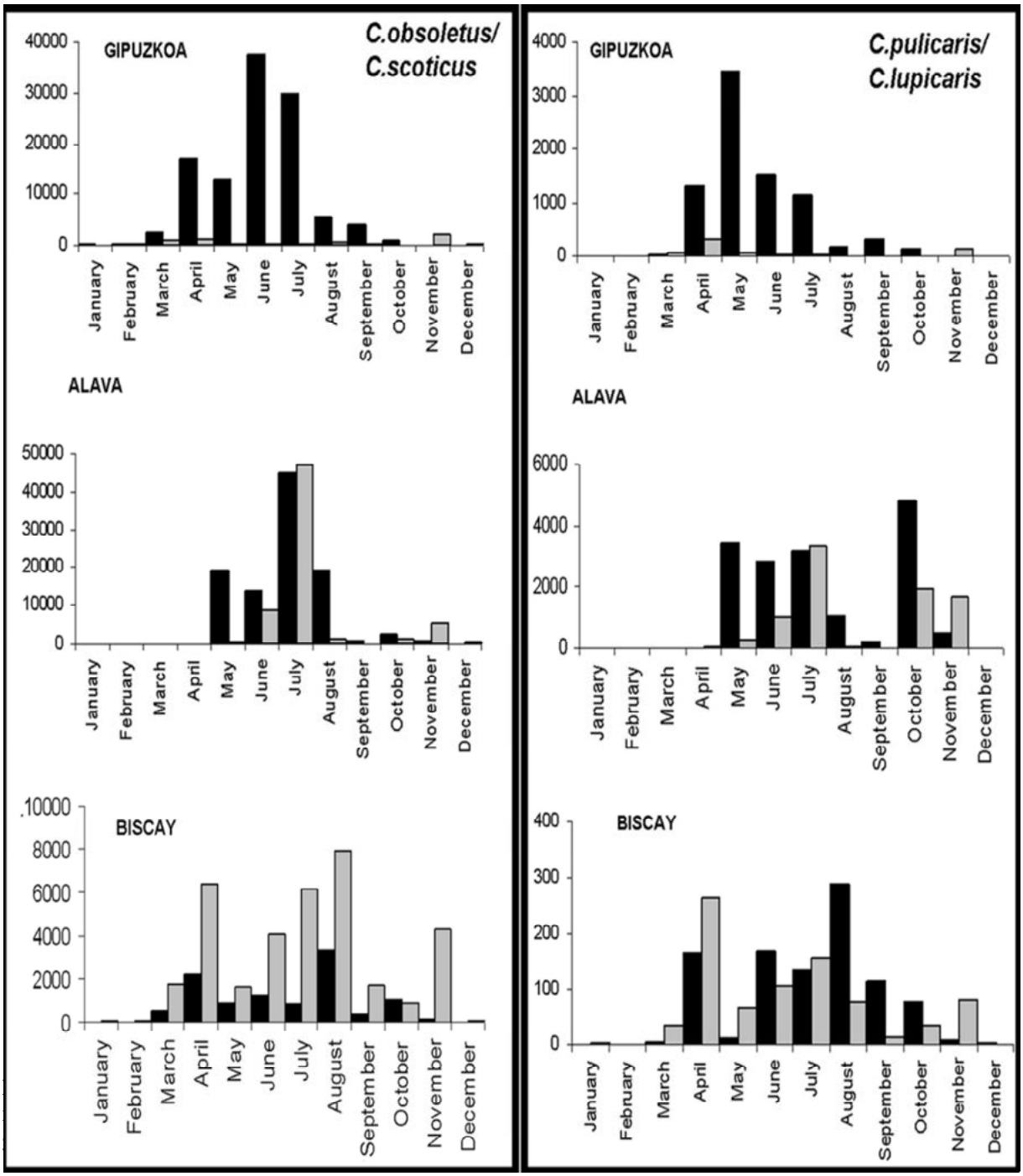


Fig. 7. Comparative outdoors (black bar) and indoors (light bar) *Culicoides* relative composition in farms according to provinces in the Basque Country (northern Spain) from 2008-2010. On the left *C. obsoletus/C. scoticus* and on the right *C. pulicaris/C. lupicaris.*

6. CONCLUSIONS

Future investigations should explore more deeply the indoor biting activities of *Culicoides*, particularly those members of the Obsoletus complex, all year round and to link the findings more conclusively to climatic factors (temperature and rainfall) and/or to husbandry practices (presence and density of animals and kind of setting i.e. size of the building and size of the opening).

The detection of few specimens of the principal Mediterranean vector of BTV *C. imicola* (in Gipuzkoa and Biscay) and several specimens of the potential vector *C. nubeculosus* (especially in Biscay) during the present study should be considered as a starting point to develop a detailed monitoring program and check the possible implication of these species in BTV transmission. It has been evident the widespread occurrence and heightened abundance of Obsoletus complex and in lesser extent the Pulicaris complex in all the sheep farms. However, low abundance levels of *C. dewulfi* and *C. chiopterus*, both implicated as vectors in northern European regions are reported. More studies are needed to improve the knowledge of both groups, focusing on biology, ecology, breeding sites and semiochemically-mediated control for Integrated Pest Management (IPM) strategies.

7. ACKNOWLEDGMENTS

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Table 3. Total (indoors + outdoors) catches of *Culicoides* species in three sheep farms from Gipuzkoa in 2008. (*): sibling species of the Obsoletus complex see details in the text. (N) number of traps, indoors + outdoors: (S) indicates the species richness and is the number of species in each month. In greyish it is highlighted the Obsoletus complex, the month with most captures and the total of *Culicoides* catches. Species ordered by subgeneric groups. In the space (S) of each column (*C. obsoletus/C. scoticus*) is referred as one species, but in (S= total) both are added. Relative data of Gipuzkoa came from the material studied by Romón et al. (2012), but we have re-examined the whole material, checking the species identification and counting again the specimens.

	GIPUZKOA	January		February		March		April		May		June		July		August		September		October		November		December		♀	♂	Total (♀+♂)	%
	(N=6)	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂				
1	*C. chiopterus*	2	0	6	2	15	36	18	10	3	16	36	4	12	11	11	16	0	7	0	1	0	0	0	0	103	103	206	0.16
2	*C. dewulfi*	0	0	0	0	0	0	0	0	0	0	0	0	10	8	6	0	0	0	2	0	0	0	0	0	18	8	26	0.02
3	*C. imicola*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0	2	0.00
4–5	*C. obsoletus/ C. scoticus**	299	32	525	48	3.227	263	18.031	241	12.299	899	36.374	1.362	29.433	984	5.268	820	4.011	505	1.046	79	2.339	23	200	17	113.052	5.273	118.325	90.70
6	*C. circumscriptus*	0	0	0	0	0	0	0	0	1	0	3	0	7	0	4	0	1	0	0	0	0	0	0	0	16	0	16	0.01
7	*C. fagineus*	0	0	0	0	0	0	0	0	0	0	2	0	11	0	7	0	1	0	0	0	0	0	0	0	21	0	21	0.02
8	*C. flavipulicaris*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
9	*C. impunctatus*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
10	*C. lupicaris*	0	0	13	1	65	4	1.370	19	3.202	162	1.145	12	1.036	8	137	12	233	80	135	8	150	0	2	0	7.488	306	7.794	5.97
11	*C. newsteadi*	0	0	0	0	0	0	23	0	173	6	78	1	28	0	15	0	0	0	1	1	0	0	0	0	318	8	326	0.25
12	*C. pulicaris*	0	1	0	0	7	0	269	4	140	11	399	0	136	0	42	1	9	4	1	0	2	0	0	0	1.005	21	1.026	0.79
13	*C. punctatus*	0	0	5	0	42	1	352	4	366	19	229	2	346	2	138	4	130	1	1	0	0	0	0	0	1.609	33	1.642	1.26
14	*C. subfagineus*	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.00
15	*C. nubeculosus*	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2	1	3	0.00
16	*C. parroti*	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	2	3	0.00
17	*C. riethi*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0.00
18	*C. stigma*	0	0	0	0	0	0	0	0	0	0	1	0	4	0	0	0	1	1	0	0	0	0	0	0	6	1	7	0.01
19	*C. cataneii*	0	0	0	0	0	0	0	0	0	0	2	0	4	0	3	0	0	0	0	0	0	0	0	0	9	0	9	0.01
20	*C. comosioculatus*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
21	*C. festivipennis*	0	0	0	0	0	0	1	0	67	7	131	9	123	13	51	25	40	6	3	3	1	0	0	0	417	63	480	0.37
22	*C. furcillatus*	0	0	0	0	0	0	0	0	1	0	3	0	8	1	3	0	0	0	0	0	0	0	0	0	15	1	16	0.01
23	*C. kibunensis*	0	0	0	0	0	0	0	0	9	1	3	0	36	5	78	1	2	0	0	0	0	0	0	0	128	7	135	0.10
24	*C. odiatus*	0	0	0	0	0	0	0	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	4	0	4	0.00
25	*C. semimaculatus*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
26	*C. achrayi*	0	0	0	0	0	0	0	0	28	0	190	5	68	8	0	0	0	0	0	0	0	0	0	0	286	13	299	0.23
27	*C. fascipennis*	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	1	0	0	0	0	0	0	0	0	5	1	6	0.00
28	*C. pallidicornis*	0	0	0	0	0	0	0	0	1	0	9	0	75	0	9	0	0	0	0	0	0	0	0	0	94	0	94	0.07
29	*C. subfasciipennis*	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0.00
30	*C. minutissimus*	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	2	0.00
	TOTAL	302	33	549	51	3.356	304	20.064	279	16.290	1.122	38.611	1.396	31.349	1.043	5.774	880	4.428	605	1.189	92	2.492	23	202	17	124.606	5.845	130.451	100.00
	(♀+♂)	335		600		3.660		20.343		17.412		40.007		32.392		6.654		5.033		1281		2515		219		130.451			
	S	4		4		5		8		13		20		25		17		11		8		4		2		30			

Table 4. Total (indoors + outdoors) catches of *Culicoides* species in five sheep farms from Alava in 2009. (*): sibling species of the Obsoletus complex see details in the text. (N) number of traps, indoors + outdoors: (S) indicates the species richness and is the number of species in each month. In greyish it is highlighted the Obsoletus complex, the month with most captures and the total of *Culicoides* catches. Species ordered by subgeneric groups. In the space (S) of each column (*C. obsoletus/C. scoticus*) is referred as one species, but in (S= total) both are added.

	ALAVA	January		February		March		April		May		June		July		August		September		October		November		December		♀	♂	Total (♀+♂)	%
	(N=10)	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂				
1	*C. chiopterus*	0	0	0	0	0	0	0	0	0	0	0	0	0	46	0	54	0	0	0	0	0	0	0	0	0	100	100	0.06
2	*C. dewulfi*	0	0	0	0	0	0	0	0	1	0	3	0	1	3	8	1	0	0	1	0	0	0	0	0	14	4	18	0.01
3–4	*C. obsoletus/ C. scoticus* *	0	0	0	0	110	10	119	16	10.071	241	15.506	145	69.037	2.073	10.827	800	561	57	2.386	55	6.063	101	506	2	115.186	3.500	11.8686	71.74
5	*C. circumscriptus*	0	0	0	0	0	0	0	1	7	1	3	1	0	1	4	1	1	0	2	0	1	0	0	0	18	5	23	0.01
6	*C. fagineus*	0	0	0	0	0	0	0	0	8	0	61	0	510	0	408	1	5	0	0	0	2	0	0	0	994	1	995	0.60
7	*C. impunctatus*	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.00
8	*C. lupicaris*	0	0	0	0	4	0	34	1	3.607	61	3.723	24	6.143	25	1.033	26	197	19	6754	40	2167	13	3	0	23.665	209	23.874	14.43
9	*C. newsteadi*	0	0	0	0	0	0	3	0	152	0	54	0	30	0	10	0	3	0	13	0	1	0	0	0	266	0	266	0.16
10	*C. pulicaris*	0	0	0	0	3	1	5	0	53	0	98	0	314	11	37	0	0	0	10	0	3	0	12	0	535	12	547	0.33
11	*C. punctatus*	0	0	0	0	2	1	0	0	1.096	1	1.554	26	1.548	40	403	12	60	4	230	4	58	1	8	0	4.959	89	5.048	3.06
12	*C. nubeculosus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0.00
13	*C. parroti*	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	2	1	1	0	0	0	3	3	7	10	0.01
14	*C. stigma*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	2	1	3	0.00
15	*C. alazanicus*	0	0	0	0	0	0	0	0	0	0	0	0	5	0	10	0	15	2	2	0	0	0	0	0	32	2	34	0.02
16	*C. brunnicans*	0	0	0	0	0	0	0	0	360	48	212	2	168	0	2	0	0	0	0	0	0	0	0	0	742	50	492	0.48
17	*C. cataneii*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	2	0	0	0	0	0	0	3	2	5	0.00
18	*C. clastrieri*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0.00
19	*C. festivipennis*	0	0	0	0	0	0	0	0	84	2	51	0	194	11	109	31	56	22	46	0	5	0	0	0	545	66	611	0.37
20	*C. furcillatus*	0	0	0	0	0	0	0	0	0	0	14	3	4.562	2	2.125	9	10	0	0	0	0	0	0	0	6.711	14	6.725	4.07
21	*C. gejgelensis*	0	0	0	0	0	0	0	0	0	0	52	6	14	0	2	0	2	0	1	0	0	0	0	0	71	6	77	0.05
22	*C. griseidorsum*	0	0	0	0	0	0	0	0	0	0	2	0	1	0	6	0	0	0	0	0	0	0	0	0	9	0	9	0.01
23	*C. heliophilus*	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.00
24	*C. kibunensis*	0	0	0	0	0	0	1	0	0	0	113	50	344	81	58	18	8	3	0	0	1	0	0	0	525	152	677	0.41
25	*C. kurensis*	0	0	0	0	0	0	0	0	0	0	2	0	6	0	18	0	0	0	0	0	0	0	0	0	26	0	26	0.02
26	*C. longipennis*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
27	*C.odiatus*	0	0	0	0	0	0	0	0	0	0	0	0	12	0	15	0	2	0	1	0	0	0	0	0	30	0	30	0.02
28	*C. paradisionensis*	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	0	4	0.00
29	*C. pictipennis*	0	0	0	0	0	0	0	0	11	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	13	0	13	0.01
30	*C. poperinghensis*	0	0	0	0	0	0	0	0	103	2	13	2	11	1	0	0	0	0	0	0	0	0	0	0	127	5	132	0.08
31	*C. santonicus*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
32	*C. semimaculatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0.00
33	*C. shaklawensis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0.00
34	*C. simulator*	0	0	0	0	0	0	0	0	4	11	27	26	116	7	4	1	0	0	0	0	0	0	0	0	151	45	196	0.12
35	*C. univitattus*	0	0	0	0	0	0	0	2	26	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	32	2	34	0.02
36	*C. vexans*	0	0	0	0	0	0	1	0	14	9	1	0	0	0	0	0	0	0	0	0	0	0	0	0	16	9	25	0.02
37	*C. achrayi*	0	0	0	0	0	0	0	0	51	2	308	4	2.164	6	228	4	1	0	0	0	0	0	0	0	2.752	16	2.768	1.67
38	*C. fascipennis*	0	0	0	0	0	0	0	0	0	0	9	0	564	0	291	55	1	0	0	0	0	0	0	0	865	55	920	0.56
39	*C. pallidicornis*	0	0	0	0	0	0	0	0	0	0	102	0	1.015	8	304	8	0	0	0	0	0	0	0	0	1421	16	1437	0.87
40	*C. picturatus*	0	0	0	0	0	0	0	0	7	0	29	2	158	0	9	0	0	0	0	0	0	0	0	0	203	2	205	0.12
41	*C. subfasciipennis*	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0.00
42	*C. minutissimus*	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	0	1	0	0	0	0	0	0	0	6	0	6	0.00
43	*C. tauricus*	0	0	0	0	0	0	0	0	2	0	14	7	911	3	188	0	0	0	0	0	0	0	0	0	1115	10	1125	0.68
	Total	0	0	0	0	119	12	163	20	15.659	378	21.963	298	87.841	2.319	16.105	1.022	924	111	9.450	100	8301	115	529	5	161.054	4.380	165.434	100.00
	(♀+♂)	0		0		131		183		16.037		22.261		90.160		17.127		1.035		9.550		8.416		534		165.434			
	S	0		0		4		8		19		28		33		30		17		14		9		5		43			

Table 5. Total (indoors + outdoors) catches of *Culicoides* species in three sheep farms from Biscay in 2010. (*): sibling species of the Obsoletus group see details in the text. (N) number of traps, indoors + outdoors: (S) indicates the species richness and is the number of species in each month., In greyish it is highlighted the Obsoletus complex, the month with most captures and the total of *Culicoides* catches. Species ordered by subgeneric group. In the space (S) of each column (*C. obsoletus/C. scoticus*) is referred as one species, but in (S= total) both are added.

	BISCAY	January		February		March		April		May		June		July		August		September		October		November		December		♀	♂	Total (♀+♂)	%
	(N=6)	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂				
1	*C. chiopterus*	0	0	0	0	2	0	1	1	0	0	6	1	1	0	3	8	0	2	0	3	2	1	0	0	15	16	31	0.06
2	*C. dewulfi*	0	0	0	0	0	0	0	0	2	1	0	7	0	0	0	3	0	3	0	0	0	3	0	0	2	17	19	0.04
3	*C. imicola*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0.00
4–5	*C. obsoletus/C. scoticus* *	46	4	95	30	2.255	33	8.402	203	2176	392	4.797	480	6112	839	9.542	1.705	1.815	262	1.819	139	4.423	54	64	12	41.546	4.153	45.699	91.62
6	*C. circumscriptus*	0	0	0	0	0	0	0	0	1	1	2	0	0	0	2	12	1	0	1	0	0	0	0	0	7	13	20	0.04
7	*C. fagineus*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.00
8	*C. impunctatus*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
9	*C. lupicaris*	1	0	0	0	36	3	411	10	72	3	237	7	253	24	206	151	94	24	68	27	83	2	2	0	1.463	251	1.714	3.44
10	*C. newsteadi*	0	0	0	0	0	0	1	1	0	4	5	0	7	0	8	2	3	2	3	1	1	0	0	0	28	10	38	0.08
11	*C. pulicaris*	0	0	0	0	1	0	3	1	1	0	8	0	6	0	4	1	6	3	4	8	0	0	0	0	33	13	46	0.09
12	*C. punctatus*	1	0	0	0	4	0	18	2	29	0	105	3	136	1	53	6	22	3	6	0	3	0	0	0	377	15	392	0.79
13	*C. nubeculosus*	0	0	0	0	0	0	0	0	0	0	13	2	7	2	21	26	7	17	3	3	0	0	0	0	51	50	101	0.20
14	*C. parroti*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	4	0	4	0.01
15	*C. stigma*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	2	3	0.01
16	*C. brunnicans*	0	0	0	0	0	0	19	14	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	14	41	0.08
17	*C. cataneii*	0	0	0	0	0	0	0	0	2	0	0	0	1	0	1	2	0	0	0	0	0	0	0	0	4	2	6	0.01
18	*C. festivipennis*	0	0	0	0	0	0	5	4	18	5	293	21	370	23	228	27	147	11	12	1	4	0	0	0	1.077	92	1.169	2.34
19	*C. furcillatus*	0	0	0	0	0	0	18	8	13	17	33	5	4	1	1	1	0	0	0	0	0	0	0	0	69	32	101	0.20
20	*C. gejgelensis*	0	0	0	0	0	0	0	1	4	0	3	0	1	0	1	0	1	0	0	0	1	0	0	0	11	1	12	0.02
21	*C. kibunensis*	0	0	0	0	0	0	0	0	9	6	131	19	110	5	63	30	8	3	2	0	0	0	0	0	326	63	389	0.77
22	*C. maritimus*	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0.00
23	*C. odiatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	5	0	5	0.01
24	*C. pictipennis*	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0.00
25	*C. poperinghensis*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
26	*C. vexans*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
27	*C. achrayi*	0	0	0	0	0	0	0	1	9	7	20	0	16	0	8	1	0	0	0	0	0	0	0	0	53	9	62	0.12
28	*C. pallidicornis*	0	0	0	0	0	0	0	0	0	0	6	1	2	1	6	1	1	0	0	0	0	0	0	0	15	3	18	0.04
29	*C. picturatus*	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0.01
30	*C. minutissimus*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0.00
	TOTAL	48	4	95	30	2.298	36	8.880	246	2.348	437	5.661	546	7.029	896	10.153	1.976	2.107	331	1.919	183	4.519	60	66	12	45.126	4.757	49.883	100.00
	(♀+♂)	52		125		2.334		9.126		2.785		6.207		7.925		12.129		2.438		2.102		45.79		78		49.883			
	S	3		1		5		12		19		16		16		18		15		12		10		2		30			

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CHAPTER 3

Version of the chapter 3 based on the following publication:

A survey of *Culicoides* developmental sites on a farm in northern Spain, with a brief review of immature habitats of European species. 2013. Veterinary Parasitology, 191 (1-2): 81-93.

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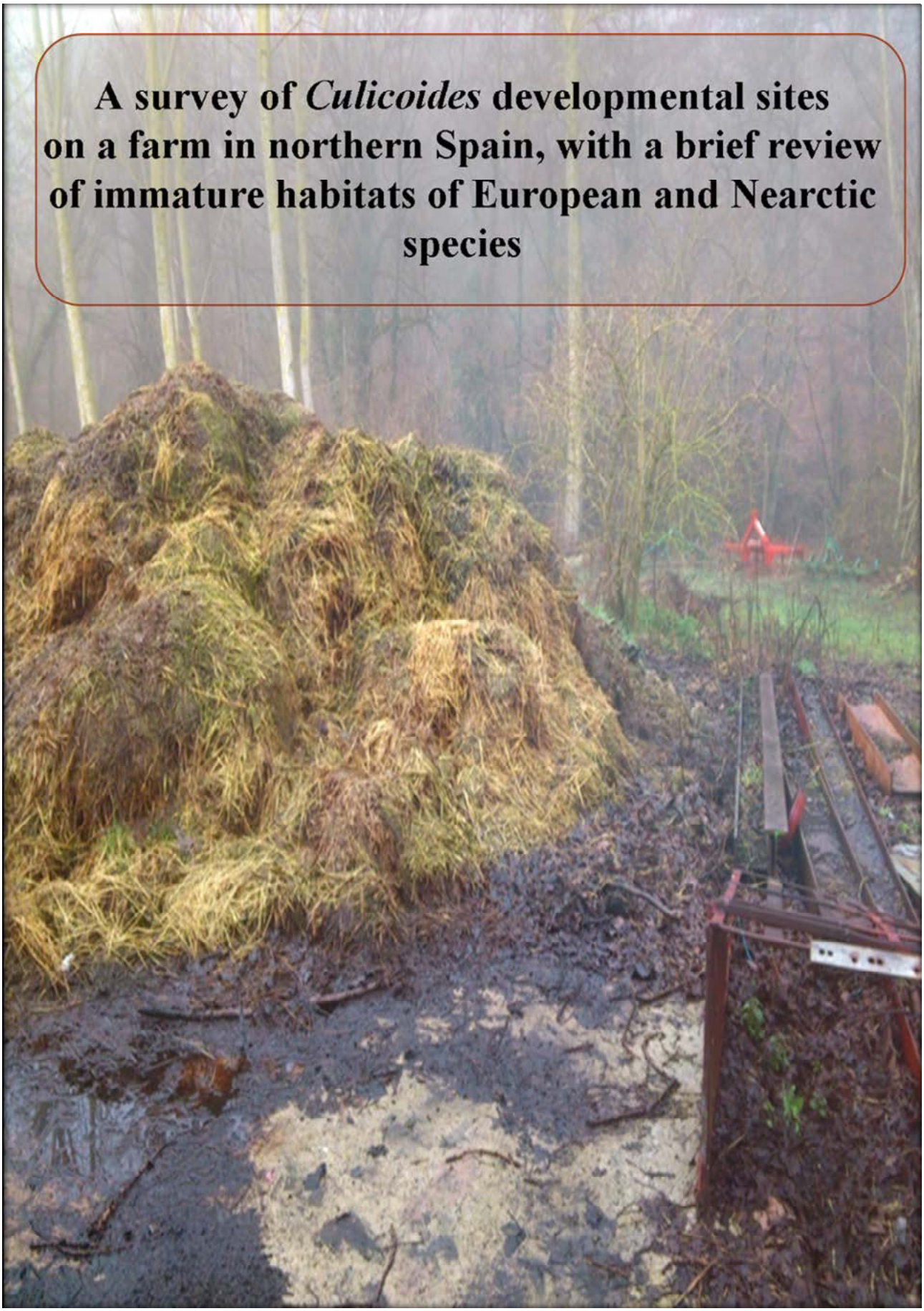
A survey of *Culicoides* developmental sites on a farm in northern Spain, with a brief review of immature habitats of European and Nearctic species



A SURVEY OF *CULICOIDES* DEVELOPMENTAL SITES ON A FARM IN NORTHERN SPAIN, WITH A BRIEF REVIEW OF IMMATURE HABITATS OF EUROPEAN AND NEARCTIC SPECIES

1. ABSTRACT

Culicoides species (Diptera: Ceratopogonidae) belonging to the Obsoletus and Pulicaris groups are considered to be the main vectors of bluetongue virus (BTV) in non Mediterranean Europe. Selected terrestrial microhabitats (n=17) on a farm in northern Spain were sampled repeatedly over a year-long period and characterized for use by *Culicoides* species for immature development. Concurrent use of CDC light traps showed the presence of 37 species and 66,569 specimens of adult *Culicoides*. A total of 28 species and 11,396 individuals emerged from laboratory-maintained soil samples. *Culicoides obsoletus* and *Culicoides scoticus* (pooled as Obsoletus complex) were particularly abundant (comprising 58.6% and 74.5% of the total collections in light traps and emergence traps respectively). Potential key vectors of animal viruses (such as BTV) were found in two main terrestrial types of microhabitats. In the case of *C. obsoletus*, different types of manure (old and composted manure, manure mixed with organic matter, and fresh manure) produced most of the specimens. In contrast, larvae of *C. scoticus* and *C. lupicaris* were associated with soil substantially comprised of rotting leaf litter that included the parasitic plant *Lathraea clandestina*. Several species, *Culicoides festivipennis*, *C. punctatus* and *C. brunnicans*, were very common in mud at pond margins. Indeed, pond microhabitats and runoff below barn rooflines supported the greatest species richness. In the pond habitat, 49.4% of *Culicoides* specimens emerged from mud at the water edge, as opposed to 50 cm above (32.4%) and 1 meter above waterline (18.2%). Similar species richness, but statistically significant differences in abundance, were observed among the four pond microhabitats. Overall, the majority of the specimens were found in the upper layer (0-3 cm), except in manure, where they preferred deeper layers (> 6 cm). Maximum peaks of abundance occurred in both light traps and soil samples in summer months, whereas increased captures in autumn were noticed only in light traps. Both trapping systems failed to collect adult *Culicoides* midges in the coldest months of December, January and February. The literature on immature habitats of species suspected in BTV transmission in Europe and North America, the Pulicaris group and particularly the Obsoletus group, is briefly reviewed.

2. KEY WORDS *Culicoides obsoletus, Culicoides scoticus, Culicoides lupicaris, Culicoides pulicaris,* Larval habitats, BTV, Basque Country

3. INTRODUCTION

The family Ceratopogonidae currently includes 6,056 species, of which 1,322 species belong to the genus *Culicoides* (Borkent, 2012). Most *Culicoides* are hematophagous, and these blood-sucking midges are particularly important as a biting nuisance to humans and to livestock as vectors of several arboviruses of domestic and wild animals. Bluetongue virus (BTV) and African Horse Sickness virus (AHSV) are both *Culicoides*-transmitted and are formally classified by (Office International des Epizooties) on the list of globally important diseases (OIE, 2012). Moreover, a new animal virus, not previously detected in Europe and initially named "Schmallenberg Virus" (SVB), is a member of the Simbu serogroup within the genus *Orthobunyavirus* (family Bunyaviridae) that are predominantly transmitted by biting midges, mainly *Culicoides* spp., and mosquitoes (Hoffmann et al. 2011; Rasmussen et al. 2012).

African horse sickness virus (AHSV) is a RNA *Orbivirus* (family Reoviridae), which causes an infection, non-contagious, disease of equids. The virus is enzootic in sub-Saharan Africa, but was present in Southern Europe (Spain) in 1956 and between 1987 and 1990 years with *C. imicola* as the main vector (Ortega et al. 1998).

BTV was reported on the Iberian Peninsula from 2000 to 2003 in the Balearic Islands and in Andalusia in 2004, after 40 years without a disease outbreak. Bluetongue disease quickly spread across the Iberian Peninsula from 2004 onwards, until November of 2007 when the virus (BTV-1) reached the Basque Country, Navarre and Atlantic Pyrenean areas, leading to 546 outbreaks. No additional outbreaks have been reported since March of 2009 in the Basque Country region (RASVE, 2012).

In Europe, three main species groups are considered as putative vectors of BTV (Meiswinkel et al. 2008). In central and northern Europe, the Obsoletus group is considered primary and includes *Culicoides obsoletus, Culicoides scoticus, Culicoides dewulfi* and *Culicoides chiopterus* (Gomulski et al. 2006; Nolan et al. 2007). Secondarily, the Pulicaris complex with *Culicoides pulicaris* and *Culicoides lupicaris* may be involved. Finally, *Culicoides imicola* is regarded as the most important vector in the southern Mediterranean

Basin and may have some role in certain locations further north (Meiswinkel et al. 2008). *Culicoides imicola* is well established in the south of Spain, but is rarely collected in the Basque Country (González et al. 2013).

In general, developmental sites of *Culicoides* species are poorly known. They can breed in a wide range of soils, if they provide enough moisture and organic matter to allow the development of the larvae (Kettle, 1962). The large range of breeding sites can be divided into three principal categories: (i) the water-saturated soil ecotone between aquatic and terrestrial habitats, (ii) dung pats (fresh dung), and (iii) moist, decaying organic matter (including manure) (Meiswinkel et al. 2004). The immature stages of *Culicoides* species usually live in surface soil layers (0-5 cm depth), and rarely are found deeper than 8 cm (Uslu and Dik, 2006). The somewhat opportunistic feeding habits of *Culicoides* larvae reflect variable food resources in their habitat (Mullen and Hribar, 1988), but they can be grouped in two categories: those which prey on small macroscopic invertebrates such as nematodes, Oligochaeta, rotifers or immature stages of insects and those which predominantly eat detritus, organic material and microorganisms such as bacteria, fungi, protozoan, yeast, diatoms and algae (Blanton and Wirth, 1979; Chaker, 1983). Habitats are known to vary within or among species in parameters such as pH, percentage of organic matter, mineral constituents and electrical conductivity. For instance *C. obsoletus/C. scoticus* are more prevalent in soil samples with a high carbon: nitrogen (C:N) ratio and high content of lignin and insoluble fibre but negatively effects was observed at higher magnesium and calcium concentrations (Braverman et al. 1974; Uslu and Dik, 2010; Zimmer et al. 2010, 2012). In terms of pH, *Culicoides* spp. have been found at pH values ranging from 4.1 to 9.4 (Smith and Varnell, 1967). Blackwell et al. (1999) and Magnon et al. (1990) noted relationships between *Culicoides* larval distribution and pH in Scottish bog and USA salt marsh habitats, respectively. Schmidtmann et al. (2000), on the other hand, noted that pH was not very useful in distinguishing immature habitats of members of the *Culicoides variipennis* complex (larvae tended to be in slightly basic conditions), while salt levels did differ significantly among habitats used by different species (Schmidtmann et al. 2000; Schmidtmann, 2006).

Knowledge of suitable breeding sites of each species, particularly those implicated in the 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. We carried out the present study to find the breeding sites of the most likely European *Culicoides* species

candidates for BTV transmission (*C. obsoletus/C. scoticus* and *C. pulicaris/C. lupicaris*) as well as the breeding places of other *Culicoides* species with unknown veterinary importance.

4. MATERIAL AND METHODS

4.1. Study area and characteristics

A farm in Barrundia, Elguea (coordinates UTM: 30T0549088, average altitude: 754 m) in the north of Alava province (Basque Country, Spain) was selected. North-central Spain is characterized by two main bio-climatic areas, and the study farm was ubicated in an oceanic climate with Eurosiberian influences. Winters are cold with intense frosts, and summers are warm and wet (average precipitation 1,200 L/m^3, average annual temperature 11 ºC; $T_{max.}$ 39 ºC and T_{min} -10 ºC). The livestock farm housed approximately 300 sheep and 15 horses and covered 3 ha, and sheep rested inside of an open stable. In front there was a wide pasture with a pond (10 m x 15 m) at the back, which was visited periodically by domestic livestock and wild fauna (e.g. foxes and wild boars). From mid-July until the first rains of September this pond was nearly dry. The farm perimeter was surrounded by a mixed and diverse forest, mainly *Quercus pyrenaica*, *Cupressus sempervirens*, *Populus alba*, *Alnus glutinosa* and *Pinus* spp. The farm also bordered a poplar grove and a river.

4.2. Sampling sites

The choice of this farm as a sampling site was based on preliminary research after a BTV-1 outbreak occurred between November 2007-October 2008 on the farm, which resulted in 30 sheep deaths. CDC-UV light traps revealed that the farm had unusually high *Culicoides* spp. abundance and diversity, so it was selected for further study.

Several suspect terrestrial farm microhabitats (n=17) were selected for *Culicoides* spp. emergence sampling as follows: (A) Outdoor fresh manure (with only a few days of fermentation, yellowish, composed of litter-straw, urine and faeces), (B) Outdoor old and composted manure (taken from a large heap near the farm, dark coloured, aged at least 1 year and with abundant earthworms and acari), (C) Farm corner (outdoor area with a mixture of manure and moist organic matter), (D) River edges (damp sand surrounding the river with sandstone structure), (E) Poplar grove soil (grass with fallen leaves, which usually remained inundated), (F) Roof runoff (area with grass just below the barn roof), (G) Forest mud

(flooded muddy soil with a humus layer in the forest), (H) Fallen leaves (moist decaying leaves, frequently utilized by the parasitic plant *Lathraea clandestina*), (I) North pond (muddy area, unvegetated, accessed by livestock to drink water), (J) West pond (area with *Juncus* spp. and *Carex* spp.), (K) East pond (standing water, with roots of *Typha* spp.), (L) South pond (vegetated area, where the predominant species was *Juncus* spp. in association with different species of the family Poaceae, (M) Manure dispersed as fertilizer in the pasture, (N) Pure sheep droppings, (O) Muddy layer from the river edge, (P) Indoor stable soil (solid litter inside sheep barn, brownish, compact, contains urine and faeces with aged less than 3 months) and (Q) Moss on the forest floor (Fig.1).

Fig. 1. Map of the study area. On dark background, the letters designate sampling sites for monitoring *Culicoides* emergence: **(A)** Fresh manure **(B)** Older manure **(C)** Corner of the farm **(D)** River edges **(E)** Poplar grove soil **(F)** Roof runoff area **(G)** Forest mud **(H)** Fallen leaves **(I)** North pond **(J)** West pond **(K)** East pond **(L)** South pond. On a grey background, sample sites with no *Culicoides* emergence: **(M)** Pasture **(N)** Sheep faeces **(O)** River mud **(P)** Sheep stable litter **(Q)** Forest moss. In the lower right corner is magnified a view of the pond. Red star indicates the CDC trap position.

4.3. Sampling period and methodology

Between January 2011 and January 2012, soil samples were taken every 2 weeks from each of the 17 sites (Fig. 1) by means of a solid iron corer (Polace-Golf, Barcelona, Spain). Each core was 20 cm in diameter, 10 cm in depth and 3141 cm^3 in total volume. Three samples were extracted in each location per sample date, separated at least 50 cm from each other. At the pond, sampling was conducted relative to the proximity of the waterline (edge, 50 cm back, and 1 m back). Additional samples in each site were taken on one date during each of the four seasons. On these dates three core samples were collected, dividing each into three different depths (0-3; 3-6; 6-9 cm). Measurements of temperature, pH, and conductivity were taken every three months (model 370 pH/mV meter with temperature CAT and model 470 portable conductivity/TDS meter, Jenway Scientific Equipment for Analysis, U.K.). Field notes about colour, humidity, granulometry, percentage of organic matter and grade of compression were also recorded every 2 weeks.

In the field, the samples were placed on dishes and covered with a plastic funnel (20 cm diameter, 22 cm high) lined with duct tape and connected to a plastic jar attached to the funnel top (Foxi and Delrio, 2010). Each collection jar was filled with a solution of propanodiol (20%), alcohol (30%) and water (50%) to preserve the specimens up to 30-35 days after sampling, when adults generally stopped emerging and were removed and stored. Instead of covering the jars with a solid top, they were sealed with Parafilm "M" (Laboratory Film, Chicago, USA), to allow gas exchange and light. In the laboratory, soil samples were maintained under ambient temperature conditions, but covered with a canvas, to avoid the rain. Specimens of adult *Culicoides* newly emerged were extracted with a pipette aspirator and stored in 99% ethyl alcohol for long term preservation.

4.4. Light trap collections

One suction light trap (CDC blacklight UV 4 W tube model 1212, Entomopraxis, Barcelona, Spain) was hung in the middle of the meadow and was readily visible from different view points. Specimens of *Culicoides* were collected into a reusable plastic jar suspended below the fan of the trap, half-filled with 1,2-propanodiol (99% purity) as an odourless collecting liquid. Light trap collections were made overnight (set at dusk and retrieved at dawn) on the same days as the microhabitat substrate sampling.

4.5. Identification

Culicoides midges were initially sorted according to their distinctive wing patterns under a stereoscopic microscope (Leica MZ95) using taxonomic keys (Delécolle, 1985; Glukhova, 2005). *Culicoides* specimens that were more difficult to identify were mounted on glass slides in Hoyer's medium (50 ml of distilled water, 30 g of gum Arabic, 200 g chloral hydrate and 20 ml of glycerin) and identified later with a Leica DM4500B microscope using additional morphological characters.

The Obsoletus complex (*C. obsoletus* and *C. scoticus* at this site) collected using light traps was pooled due to the inherent difficulty in separating thousands of extremely similar specimens in different species. A sub-sample of them was examined more carefully and was identified to species level according to the morphological features noted by Augot et al. (2010) and Nielsen and Kristensen (2011).

4.6. Data analysis

Culicoides mean abundances in both pond and manure habitats were analysed by one-way parametric ANOVA followed by post hoc multiple comparisons. Contrasts were performed at a significance level of α=0.05 (SPPS for Windows). Data from manure habitats first were subjected to a log (x + 0.5) transformation, in order to remove heteroscedasticity. For the four most common *Culicoides* species in the pond, parametric ANOVA was sometimes used. A nonparametric Kruskal-Wallis analysis, followed by Mann-Whitney U-test, was conducted when no normality and homoscedasticity could be assumed. Data are expressed in terms of means ± standard error.

5. RESULTS

5.1. Light trap and soil sample collections

A total of 66,569 *Culicoides* belonging to 37 species was captured by means of light traps, whereas 11,396 specimens and 28 species were recovered from soil samples (Tables 1-2). In both cases, the Obsoletus complex (*C. obsoletus/C. scoticus*) was predominant, accounting for 58.6% of *Culicoides* specimens in light traps and 74.5% in soil samples. On the other hand, the Pulicaris complex (*C. pulicaris/C. lupicaris*) comprised 20.9% of the

total catches in the light traps, but they comprised only 1.2% of *Culicoides* midges recovered from soil samples. The remaining species are shown in Tables 1-2. The breeding sites of nine species remain unknown at this location. Only *Culicoides duddingstoni* and *Culicoides maritimus* were present in the soil samples and not in the CDC traps.

5.2. Larval habitats

Substantial numbers of adult *Culicoides* emerged from terrestrial habitats; means below are presented with letters designating statistically significant differences. Old, composted manure produced 42.4% of the total *Culicoides* midges collected (535.44 ± 177.94 a adults per sample) followed by the farm corner 19.5% (240.10 ± 89.33 b) and fresh manure 7.5% (84.50 ± 45.55 c) ($F_{2;55}$=21.84, P<0.001). In the four different pond habitats, *Culicoides* spp. were abundant in the west pond-reed bed (8.5%, 162.16 ±19.60 a) and east pond-bulrush areas (6.2%, 116.66 ± 25.61 a). In contrast, the north pond-mud unvegetated (2.8%, 54.00 ± 10.96 b) and south pond (1.8%, 33.50 ± 11.50 b), were less productive ($F_{3;44}$=11.12, P<0.001) (Fig. 2, Table 4).

Of the whole range of potential breeding sites, five sites produced no *Culicoides* midges: moss of the forest, manure dispersed as fertilizer in the pasture, pure sheep droppings, soil inside the sheep-stable and a strictly muddy layer (without sandy structure) near the river edge (microhabitat O, Fig. 1). Other river's edge habitats, featuring sand plus some organic materials, did yield some *Culicoides* (microhabitat D, mentioned as "river edges" hereafter, Fig. 1).

According to the breeding site collections, *C. obsoletus* was the most common species in old manure, in the farm corner, and in fresh manure, where they comprised more than 99% of adults emerging from the samples (Table 4). The remaining 1% of midges in manure included *C. scoticus* and *C. lupicaris* and other three minority *Culicoides* species. In contrast, *C. scoticus* accounted for 92% of *Culicoides* emerging from fallen dead leaves and 21% of *Culicoides* emerging from soil in the poplar grove. The Pulicaris complex was not dominant in any microhabitat, but substantial collections were recorded emerging from the fallen leaves (38.4% of captures), and less commonly from the forest mud and river edges (Table 4).

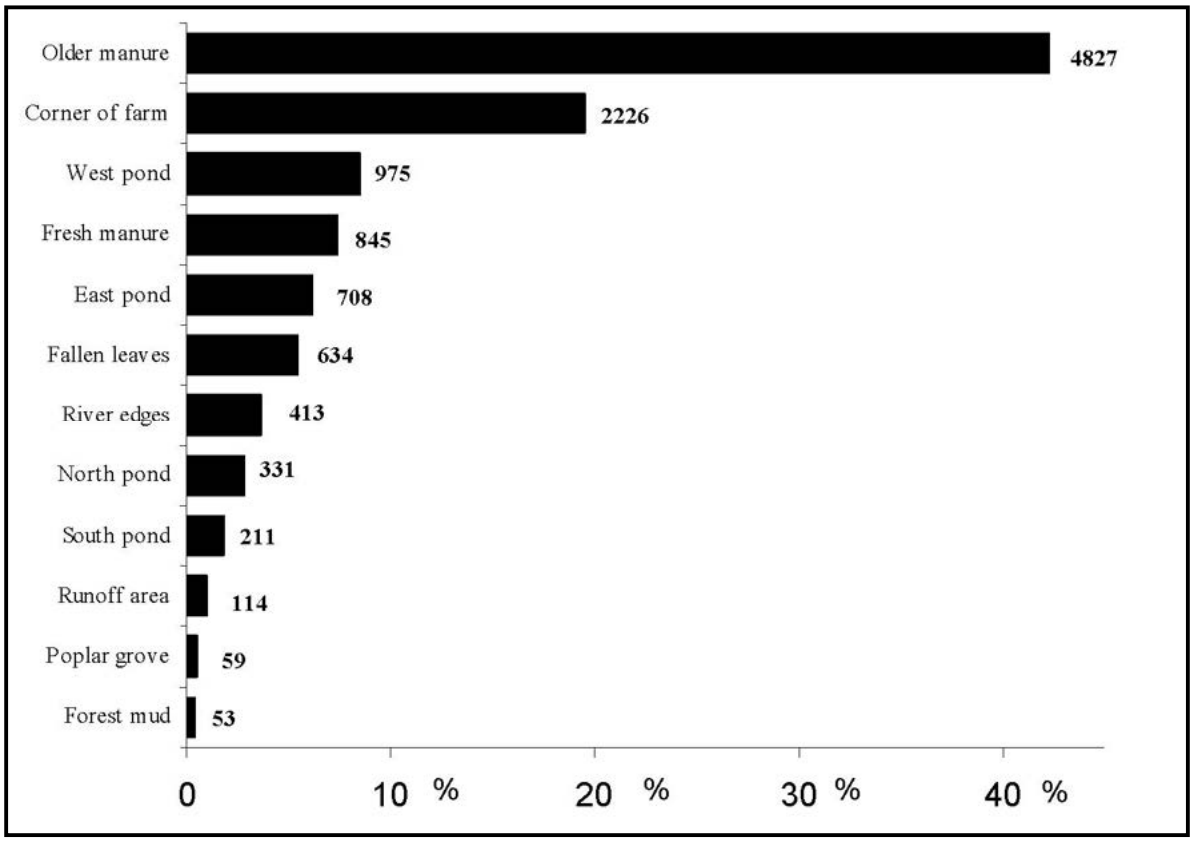


Fig. 2. The total collections of *Culicoides* specimens and their relative abundance recorded from 12 soil sampling locations in the farm and pond from Elguea (Alava province, Basque Country).

Other species of unknown economic importance, such as *Culicoides festivipennis,* were most prevalent along three margins of the pond (north, west and east) together with *Culicoides punctatus, C. brunnicans* and *C. circumscriptus.* Significant differences in the abundance of these species were found in the pond margins, as shown in Table 3. In contrast, along the river edges and in the forest mud, *C. kibunensis* was the common species.

5.3. Vertical and horizontal distribution

The results of depth collections indicated that the majority of the specimens occupied the uppermost layer (0-3 cm). There were a few exceptions; in the muddy area of the pond (north pond) many individuals emerged from the 3-6 cm layer, and curiously many *Culicoides* midges emerged from deeper layers in fresh manure, as deep as 9-12 cm or more.

Collections at different waterline distances showed the highest numbers near the waterline (49.4% of the catches) versus 50 cm (32.4%) and 1 meter above waterline (18.2%).

5.4. Seasonal collections

The pattern of abundance peaks corresponded with the summer months. CDC trap collections peaked in July and August, and emergence from soil samples peaked in June. No *Culicoides* specimens were recorded by either collection method during the coldest months between December and March. In March, April and November occasional individuals of Obsoletus and Pulicaris group midges were recorded both in light traps and emerging from soil samples. According to the light trap catches of the most common species, most reached their maximum populations in July, except *Culicoides fagineus* and *C. pallidicornis* (August), *C. newsteadi*, *C. brunnicans* and *C. lupicaris* (May). *Culicoides obsoletus/C. scoticus* increased from May to July and then decreased progressively, but with a small additional peak in October. This was very similar to *Culicoides lupicaris*, but for this one the autumn peak was greater than the summer one. While *Culicoides lupicaris*, *C. furcillatus* and *C. achrayi* were very common in light traps, few emerged from terrestrial soil samples.

Table 1. Number of *Culicoides* (females and males pooled) caught with a CDC-UV trap from Elguea (Alava, Basque Country), during a year-long sampling effort (January 2011-2012).

	Species (CDC)	Januar	Febru	March	April	May	Jun	July	August	Septem	Octobe	Novemb	Decemb	Total	%
a	*C. chiopterus*	0	0	0	0	0	0	46	53	0	0	0	0	99	0.1
a	*C. dewulfi*	0	0	0	0	0	0	2	8	0	1	0	0	11	0.0
a	*C. obsoletus/C.scoticus*	0	0	2	11	4234	3048	21512	8709	235	1049	236	0	39036	58.6
b	*C. circumscriptus*	0	0	0	0	0	0	1	3	0	0	0	0	4	0.0
c	*C. fagineus*	0	0	0	0	0	37	177	306	1	0	0	0	521	0.8
c	*C. impunctatus*	0	0	0	0	0	2	0	0	0	0	0	0	2	0.0
c	*C. lupicaris*	0	0	0	5	2961	1587	2631	949	151	4823	453	0	13560	20.4
c	*C. newsteadi*	0	0	0	0	143	34	28	2	0	6	0	0	213	0.3
c	*C. pulicaris*	0	0	0	0	11	0	302	28	0	0	0	0	341	0.5
c	*C. punctatus*	0	0	1	0	920	753	1081	304	26	149	4	0	3238	4.9
d	*C. parroti*	0	0	0	0	0	0	2	0	0	1	0	0	3	0.0
d	*C. stigma*	0	0	0	0	0	0	0	1	0	1	0	0	2	0.0
e	*C. alazanicus*	0	0	0	0	0	0	5	10	0	0	0	0	15	0.0
e	*C. brunnicans*	0	0	0	0	223	84	129	2	0	0	0	0	438	0.7
e	*C. clastrieri*	0	0	0	0	0	0	0	1	0	0	0	0	1	0.0
e	*C. festivipennis*	0	0	0	0	11	11	145	106	39	22	0	0	334	0.5
e	*C. furcillatus*	0	0	0	0	0	9	1738	1964	1	0	0	0	3712	5.6
e	*C. gejgelensis*	0	0	0	0	0	0	0	1	0	1	0	0	2	0.0
e	*C. griseidorsum*	0	0	0	0	0	0	0	6	0	0	0	0	6	0.0
e	*C. kibunensis*	0	0	0	0	0	1	68	46	0	0	0	0	115	0.2
e	*C. kurensis*	0	0	0	0	0	2	5	18	0	0	0	0	25	0.0
e	*C. longipennis*	0	0	0	0	0	0	1	1	0	0	0	0	2	0.0
e	*C. odiatus*	0	0	0	0	0	0	12	6	2	1	0	0	21	0.0
e	*C. paradisionensis*	0	0	0	0	0	0	4	0	0	0	0	0	4	0.0
e	*C. pictipennis*	0	0	0	0	11	0	1	0	0	0	0	0	12	0.0
e	*C. poperinghensis*	0	0	0	0	6	3	4	0	0	0	0	0	13	0.0
e	*C. santonicus*	0	0	0	0	0	0	1	0	0	0	0	0	1	0.0
e	*C. shaklawensis*	0	0	0	0	0	0	0	1	0	0	0	0	1	0.0
e	*C. simulator*	0	0	0	0	4	32	106	4	0	0	0	0	146	0.2
e	*C. univitattus*	0	0	0	0	13	1	0	0	0	0	0	0	14	0.0
e	*C. vexans*	0	0	0	0	0	1	0	0	0	0	0	0	1	0.0
f	*C. achrayi*	0	0	0	0	51	281	1769	226	0	0	0	0	2327	3.5
f	*C. fascipennis*	0	0	0	0	0	8	419	304	1	0	0	0	732	1.1
f	*C. pallidicornis*	0	0	0	0	0	0	201	302	0	0	0	0	503	0.8
f	*C. picturatus*	0	0	0	0	0	21	119	8	0	0	0	0	148	0.2
g	*C. tauricus*	0	0	0	0	0	4	785	179	0	0	0	0	968	1.5
	Total	0	0	3	16	8588	5916	31297	13552	456	6054	693	0	66569	100

Species ordered by subgenus groups: **(a)** *Avaritia* **(b)** *Beltranmyia* **(c)** *Culicoides* **(d)** *Monoculicoides* **(e)** *Oecacta* **(f)** *Silvaticulicoides* and **(g)** *Pontoculicoides.*

Table 2. Number of *Culicoides* (females and males pooled) emerging from breeding sites from Elguea (Alava province, Basque Country), during a year-long sampling survey (January 2011-2012). Sex ratio (female: male) shown when the total catch n≥100.

	Species (breeding sites) / sex ratio	January	February	March	April	May	June	July	August	September	October	November	December	Total	%
a	*C. dewulfi*	0	0	0	0	6	0	0	0	0	0	0	0	6	0.1
a	*C. obsoletus/C. scoticus* (1.05/ 1.02)	0	0	29	47	565	4012	1828	1409	287	228	83	0	8488	74.5
b	*C. circumscriptus* (1.10)	0	0	0	0	4	43	99	15	4	0	2	0	167	1.5
c	*C. lupicaris* (1.08)	0	0	7	5	0	18	65	19	5	10	1	0	130	1.1
c	*C. newsteadi*	0	0	0	0	0	21	16	0	0	0	0	0	37	0.3
c	*C. pulicaris*	0	0	2	3	0	2	0	1	1	0	0	0	9	0.1
c	*C. punctatus* (1.08)	0	0	3	3	5	131	100	58	13	8	0	0	321	2.8
d	*C. parroti*	0	0	0	0	0	0	4	0	0	0	0	0	4	0.0
e	*C. alazanicus*	0	0	0	0	0	2	1	2	0	0	0	0	5	0.0
e	*C. brunnicans* (1.07)	0	0	1	97	156	31	3	0	0	0	0	0	288	2.5
e	*C. cataneii*	0	0	0	0	0	0	1	0	0	0	0	0	1	0.0
e	*C. festivipennis* (1.09)	0	0	0	4	18	14	189	381	330	0	0	0	936	8.2
e	*C. furcillatus*	0	0	0	0	3	1	7	0	0	0	0	0	11	0.1
e	*C. duddingstoni*	0	0	0	0	0	3	0	17	0	0	0	0	20	0.2
e	*C. gejgelensis*	0	0	0	0	0	0	2	0	0	0	0	0	2	0.0
e	*C. kibunensis*(08.1)	0	0	0	0	171	33	114	50	2	2	0	0	372	3.3
e	*C. maritimus*	0	0	0	0	0	1	0	0	0	0	0	0	1	0.0
e	*C. pictipennis*	0	0	3	5	11	1	0	0	0	0	0	0	20	0.2
e	*C. poperinghensis*	0	0	0	0	1	2	6	0	0	0	0	0	9	0.1
e	*C. santonicus*	0	0	0	5	20	8	3	0	0	0	0	0	36	0.3
e	*C. simulator*	0	0	0	3	31	41	1	0	0	0	0	0	76	0.7
e	*C. vexans* (1.05)	0	0	0	127	38	17	0	0	0	0	0	0	182	1.6
f	*C. achrayi*	0	0	0	3	18	47	13	1	1	0	0	0	83	0.7
f	*C. fascipennis*	0	0	0	0	17	6	3	1	0	0	0	0	27	0.2
f	*C. pallidicornis*	0	0	0	0	0	0	3	0	0	0	0	0	3	0.0
f	*C. picturatus* (1.08)	0	0	1	6	45	27	55	0	0	0	0	0	134	1.2
g	*C. tauricus*	0	0	0	0	0	14	11	3	0	0	0	0	28	0.2
	Total	0	0	46	308	1109	4475	2524	1957	643	248	86	0	11396	100

Species ordered by subgenus groups: **(a)** *Avaritia* **(b)** *Beltranmyia* **(c)** *Culicoides* **(d)** *Monoculicoides* **(e)** *Oecacta* **(f)** *Silvaticulicoides* and **(g)** *Pontoculicoides*.

Table 3. Mean abundance (± SE) of the four predominant *Culicoides* species from the pond margins in Elguea (Basque Country). Pond section **(J)** featured *Juncus* and *Carex* spp. **(K)** had *Typha* spp. roots **(I)** was unvegetated and accessed by livestock, and **(L)** had *Juncus* and Poaceae family plants. Mean number emerged/sample ± SE.

Species	Pond J	Pond K	Pond I	Pond L
C. circumscriptus	4.07 ± 0.81 a	7.92 ± 1.03 b	0.35± 0.16 c	0 ± 0 c
C. punctatus	7.14 ± 0.91 a	9.85 ± 0.77 a	0.28 ± 0.12 b	0.92 ± 0.28 b
C. brunnicans	10.14 ± 1.56 a	0 ± 0 b	6.92 ± 0.99 a	2.14 ± 0.94 c
C. festivipennis	33.50 ± 6.61 a	22.00 ± 1.49 a	10.50 ± 2.49 b	0 ± 0 c

Different letters in a row indicate significant differences in abundance, at the 5% level.

Table 4. Number of total *Culicoides* captures in 12 soil sampling locations (A-H, farm microhabitats; I-L, pond microhabitats). Capital letters correspond with habitats labeled in figure 1.

	Species	Farm microhabitats								Pond microhabitats			
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
a	*C. dewulfi*	0	6	0	0	0	0	0	0	0	0	0	0
a	*C. obsoletus*	845	4790	2219	2	0	2	4	0	1	0	0	2
a	*C. scoticus*	0	17	3	2	11	4	0	584	0	0	0	2
b	*C. circumscriptus*	0	0	0	0	0	1	0	0	5	60	101	0
c	*C. lupicaris*	0	12	4	21	5	14	23	50	0	1	0	0
c	*C. newsteadi*	0	0	0	0	0	0	0	0	0	20	17	0
c	*C. pulicaris*	0	1	0	0	0	4	0	0	0	0	4	0
c	*C. punctatus*	0	0	0	0	0	60	0	0	4	101	143	13
d	*C. parroti*	0	0	0	0	0	0	0	0	4	0	0	0
e	*C. alazanicus*	0	0	0	0	0	0	0	0	2	1	2	0
e	*C. brunnicans*	0	0	0	0	14	11	0	0	90	142	1	30
e	*C. cataneii*	0	0	0	0	0	1	0	0	0	0	0	0
e	*C. festivipennis*	0	1	0	0	8	5	0	0	147	467	308	0
e	*C. furcillatus*	0	0	0	7	0	0	3	0	0	0	0	1
e	*C. duddingstoni*	0	0	0	0	0	0	0	0	6	0	14	0
e	*C. gejgelensis*	0	0	0	0	0	0	0	0	0	2	0	0
e	*C. kibunensis*	0	0	0	353	0	3	13	0	0	0	3	0
e	*C. maritimus*	0	0	0	0	0	0	0	0	0	1	0	0
e	*C. pictipennis*	0	0	0	0	0	0	0	0	0	8	11	1
e	*C. poperinghensis*	0	0	0	0	0	0	0	0	1	2	0	6
e	*C. santonicus*	0	0	0	0	0	1	0	0	4	9	16	6
e	*C. simulator*	0	0	0	0	0	5	0	0	2	43	3	23
e	*C. vexans*	0	0	0	0	0	1	0	0	58	96	11	16
f	*C. achrayi*	0	0	0	0	6	1	7	0	3	9	8	49
f	*C. fascipennis*	0	0	0	0	8	0	0	0	3	12	0	4
f	*C. pallidicornis*	0	0	0	0	0	0	3	0	0	0	0	0
f	*C. picturatus*	0	0	0	0	7	1	0	0	1	1	66	58
g	*C. tauricus*	0	0	0	28	0	0	0	0	0	0	0	0
	Σ species [a]	**1**	**6**	**3**	**6**	**7**	**15**	**6**	**2**	**15**	**16**	**15**	**13**

Species ordered by subgenus groups: **(a)** *Avaritia* **(b)** *Beltranmyia* **(c)** *Culicoides* **(d)** *Monoculicoides* **(e)** *Oecacta* **(f)** *Silvaticulicoides* and **(g)** *Pontoculicoides*. [a] Total number of *Culicoides* species collected per type of microhabitat.

6. DISCUSSION

6.1. Species richness

Culicoides species in the Basque Country were almost unknown until November 2007, when a monitoring program was implemented on some farms due to bluetongue outbreaks which occurred in Gipuzkoa province (Goldarazena et al. 2008; Romón et al. 2012). Subsequently, other entomological surveys were conducted with CDC-UV traps on sheep farms from the other two provinces (Alava and Biscay) of the Basque Country, demonstrating the presence of 49 *Culicoides* species (González and Goldarazena, 2011). Supplementary studies in natural habitats allowed addition of three more species, bringing the total of 52

species of *Culicoides* for the Basque Country territory. In this previous study we confirmed the widespread abundance of the Obsoletus group on all the farms, showed the Pulicaris group is common, and collected some specimens of *C. imicola* and several *C. nubeculosus*, particularly in the northeast of the Basque Country. The presence of 37 species on a single farm in the current study provided a remarkably good representation of the entire Basque region *Culicoides* fauna.

6.2. Breeding sites of the Obsoletus group

The subgenus *Avaritia* in Spain is comprised of six species: *C. obsoletus, C. scoticus, C. dewulfi, C. chiopterus, C. imicola* and *C. montanus.* Five of them have been reported in the Basque Country and four in this study. Their role as potential vectors of BTV is well-documented, based on abundance, propensity to bite ruminants, and BTV detection from insect pools (De Liberato et al. 2005; Savini et al. 2005; Hoffmann et al. 2009; Mehlhorn et al. 2007; Dijkstra et al. 2008; Gerry et al. 2009; Romón et al. 2012) or in laboratory competence studies (Carpenter et al. 2006, 2008a). Further studies are necessary to elucidate the role of *Culicoides montanus* in the potential transmission of BTV (Foxi et al. 2011); it is a very uncommon member of the Obsoletus group (not yet found in the Basque country).

Records of the breeding sites of the Obsoletus complex are numerous, sometimes superficial, and frequently confused because older studies pooled Obsoletus s.l. species. Overall, *C. obsoletus* s.s. in the Palearctic appears to utilize a wide range of habitats, including moist forest leaf litter, tree holes (Kremer, 1965) standing water and marsh edges with vegetation (Dzhafarov, 1964, 1976), swamps, rotting vegetation, water-filled tree holes, feces-contaminated straw, organically enriched soil in stable yards, and wet soil rich in organic matter in shaded habitats; it seems they do not prefer to breed in waterlogged soils (Hill, 1947; Kettle and Lawson, 1952; Weinburg and Pratt; 1962; Braverman et al. 1974; Trukhan, 1975; Mirzaeva et al. 1976, Mellor and Pitzolis, 1979; Mathieu, 2005; Zimmer et al. 2010, 2013; Ninio et al. 2011). In America, *Culicoides obsoletus* developmental sites similarly range rather broadly. They have been reared from soils rich in organic humus (e.g. sphagnum/peat) and/or manure of various types in the soil (Battle and Turner, 1971; Mullen and Hribar, 1988), in the top few centimeters from straw in chicken coops, blended with different materials such as wood chips and feathers (Jamnback and Wirth, 1963; Hair et al. 1966), in decomposing cornstalks, an old manure pile, a low hay pile, and manure-bedding mixtures on Wisconsin dairy farms (Jones, 1961), and from similar habitats in New York state (Jamnback, 1965).

Recent studies show that *C. obsoletus* develop in barn roof (runoff areas) surrounding stables (Mathieu, 2005), may occur together with *C. scoticus* larvae in maize silage residues, and can be found in fairly dry dung adhering to walls inside enclosures (Zimmer et al. 2010). Ninio et al. (2011) recorded numerous adults of *C. obsoletus* emerging from soil samples including significant cattle manure (both collected indoors and outdoors). Their larvae have been also found in horse dung (IAH, 2010). This plasticity in habitat utilization by *C. obsoletus* could explain their ubiquitous distribution across Europe and development in a great variety of habitats. On the other hand, *C. scoticus* s.s. breeding sites have been largely unknown (Kettle and Lawson, 1952), but they have been found breeding on rotting fungi (Buxton, 1960), and marshy habitats of southern England (Boorman and Goddard, 1970). As shown in the present study, their immature habitats actually are not this restricted, and they may coexist with *C. obsoletus* fairly regularly (Conte et al. 2007).

In the current study *C. obsoletus* s.s. was captured in great numbers both in light traps and emerging from soil samples, where it was the predominant species. The majority of the *C. obsoletus* specimens were collected in heaps of old-composted manure (3,225 females, 1,565 males [60.9% of the total *C. obsoletus* soil collections]). This was followed by the farm corner (1,760 females, 459 males [28%]), fresh manure, (385 females, 460 males [10.6%]) and the remaining specimens (less than 1%) were found developing in other five microhabitats. As expected, catches from light traps showed a clear female-biased sex ratio, as females frequently are more mobile, range more widely for a proper host for blood feeding, and are highly attracted to light traps (Venter and Hermanides, 2006; Venter et al. 2009). In contrast, the sex ratio found in emergence traps was more balanced.

The sex ratio (♀/♂) of the total *C. obsoletus* in the present study (emerging from soil samples) was 1.05, very similar to 1.06 calculated by Ninio et. al. (2011) from outdoor-indoor *Culioides obsoletus* s.s. recovered from soil samples. The greatest abundance was found in a soil sample of old manure, in which 832 females and 418 males of *C. obsoletus* emerged from 314 cm^2 of surface area. Old manure (1 year old) was characterized by fairly stable humidity, high biological activity (abundant earthworms and Acari), 45% organic matter, pH values between 8.5 and 8.7 and electrical conductivity between 2.6 and 9.6 µS/cm. These microhabitats seem to be the most favourable breeding site for *C. obsoletus* s.s.

The other abundant member of the Obsoletus complex, *C. scoticus*, was commonly collected in light traps, and significant collections also were made from soil samples. Based on light traps, *C. scoticus* was eight times less abundant than *C. obsoletus* (González et al. 2013). They were by far most common emerging from fallen leaf habitats (483 females and 101 males [93.7%]); the remaining specimens emerged from other six microhabitats (Table 4). The sex ratio (♀/♂) of emerged *C. scoticus* was 1.02. In light of the results, *C. scoticus* is unlikely to breed in great numbers in manure or manure-laden habitats. Only 20 specimens were captured in the spring. Interestingly, the majority of the adult emergence occurred associated with an organic layer where the plant *Lathraea clandestina* grows. So, this should be further investigated as a possible indicator organism for presence of *C. scoticus*, although there is not necessarily a direct connection. Interestingly, habitats reported for immature *Culicoides* (*Avaritia*) *sanguisuga* (an American species very similar morphologically to *C. scoticus*) was "highly localized and specific" in New York State (Jamnback, 1965). Such habitats consisted of leaf accumulations on well-drained slopes (especially beech trees, *Fagus grandifolia*); leaves were wet ("enough moisture to glisten") underneath but dry on the surface. It is tempting to hypothesize that this biological similarity may be related to the morphological similarity for both species. The pH levels in *C. scoticus* habitats in the present study typically varied from 7.5 to 7.7, with electrical conductivity between 196 and 202 µS/cm in the fallen leaves. In contrast, values of pH ranged from 8 to 8.1 with conductivity of 1681 and 1742 µS/cm in plant root zones.

In contrast to Zimmer et al. (2010) and Ninio et al. (2011) who found *C. obsoletus/C. scoticus* and *C. obsoletus* developing in the relatively dry bedding litter on walls and in the corner angles inside cowsheds respectively, we do not report any specimens developing in the upper 15 cm of the indoor central part of the sheep stables. The measurements of pH and conductivity inside the sheds showed conditions were quite basic, ranging from pH 9 to 9.2. Perhaps this high alkalinity, together with the trampled soil, creates a compacted mass which is non-permissive to larval movement, and therefore no larvae were encountered. Ninio et al. (2011) described the indoor samples as dark compost topped with a layer of dry substrate and loose in consistency, which differs from stable soil in the present study.

Moreover, exceptional samples collected from slurry manure deposited on heap margins (dense liquid, black, 3-cm thickness) resulted also in no emergence of *Culicoides*, but was favourable for immense number of other dipterans: families Sphaeroceridae and Syrphidae predominantly.

Additionally, it is worthwhile to mention that some outdoor samples of soil with manure from horses and cows resulted in some *C. obsoletus* emergence in the present study.

Overall, it seems that both *C. obsoletus* and *C. scoticus* are able to breed in a great variety of habitats, but they show distinct preferences for certain soil conditions. In addition, we observed that Obsoletus group larvae probably are incompatible with flooded soils, showing preferences for unsaturated, anthropogenic soil environments in the vicinity of the farms (Hill, 1947; Zimmer et al. 2008b). Meiswinkel et al. (2004) also reported that the pupae of most species in the subgenus *Avaritia* cannot float and usually drown when submerged in water.

Culicoides chiopterus and *C. dewulfi*, both potential vectors of BTV, have been reared from cattle or horse dung (Kettle and Lawson, 1952). *Culicoides chiopterus* have also been found in sheep droppings, in fungi, in sap from elm trees and from bogs with rotting vegetation (Kettle and Lawson, 1952; Campbell and Pelham-Clinton, 1960; Dzhafarov, 1964). Recently, Dijkstra et al. (2008) pointed out that this species breeds exclusively on dung of livestock; no specimens were captured emerging from soil samples in the present study, although some specimens were collected as adults by using CDC-traps. *Culicoides dewulfi* was reported developing in cow dung in England (Kettle and Lawson, 1952; Campbell and Pelham-Clinton, 1960), in France (Kremer, 1965) and in Belgium (Zimmer et al. 2008a). In the present study, a total of six male specimens of *C. dewulfi* were captured emerging from older heaps of manure in May, and 11 (nine females and two males) adults were taken in light traps.

The males of both species (e.g. *chiopterus* vs. *dewulfi*) are easily distinguished based on the presence of three main morphological characters, which include the presence/absence of a spiculate membrane, presence/absence of apodemes, and size of the *aedeagus* membrane (Delécolle, 1985). In contrast females of certain members of the Obsoletus group (i) could be underestimated, (ii) be less attracted to UV-traps or (iii) be mistaken for other members of the same group (Carpenter et al. 2008b). Newly emerged (teneral) adults, for which wing patterns were relatively pale as they drowned in fluid in collecting jars, were especially difficult to differentiate. Even so, the number of specimens of *C. chiopterus* and *C. dewulfi*, in comparison with *C. obsoletus* s.l., seems to be perhaps artificially low relative to some other studies carried out in Northern Europe (Baldet et al. 2008; Ander et al. 2011).

Some members of the Obsoletus group (*C. obsoletus* and *C. chiopterus*) appear to have Holarctic distributions, ranging from western Europe, into Manchuria and northern Asia (Arnaud, 1956), north Africa and fully across north America, from Alaska south to California, east through much of Canada and south to Georgia (Wirth et al. 1985).

This remarkable range unfortunately is based overwhelmingly on adult midge morphology, which can be problematic in this group, and especially in the females which are more likely to be encountered. Murphree and Mullen (1991) noted substantial variation in larval morphology of *C. obsoletus*, e.g. in larval comb structure on the epipharynx, in various locations in the Palearctic and Nearctic. This may reflect taxonomic differences and feeding habits. A comprehensive taxonomic approach, incorporating aspects such as immature morphology and application of molecular tools, is badly needed across the full geographic range to address the true identity and especially the Asian and Nearctic distributions of these species. Jamnback and Wirth (1963) noted further that the dominant eastern North American forest species *C. sanguisuga* strongly resembles the European *C. scoticus*, differing mainly in size (*C. scoticus* is larger) and the extent of division along the 9th abdominal sternum of males. Even that feature shows some plasticity, and they reported seeing two males from Minnesota, USA that matched *C. scoticus*.

Culicoides chiopterus is the other Obsoletus group member that is found in North America; Wirth et al. (1985) simply list its range as the entire Canada and USA. The larvae are not too difficult to distinguish from those of *C. obsoletus*, due to the smaller, pale-yellow head capsule (Murphree and Mullen, 1991). Less is known of *C. chiopterus* immature habitats in the Nearctic. Manure and manure polluted habitats (straw and moist soil polluted with chicken manure) are the most commonly reported sites for immatures of *C. chiopterus* in America (Jamnback, 1965).

6.3. Breeding sites of the Pulicaris group

The subgenus *Culicoides* in Spain includes ten species: *C. almeidae, C. lupicaris, C. pulicaris, C. punctatus, C. newsteadi, C. fagineus, C. flavipulicaris, C. impunctatus, C. subfagineus* and *C. deltus*, and all of them, with the exception of *C. almeidae* and *C. deltus*, are present in the Basque Country. Two species of these are included in the Pulicaris complex (*C. pulicaris* and *C. lupicaris*) of which *C. pulicaris* is of particular concern due to the

isolation of BTV in parous specimens collected in the field (Mellor et al. 1990; Caracappa et al. 2003; Romón et al. 2012). Both species, whose taxonomy is still controversial among specialists, usually can be morphologically distinguished by their wing pattern.

The larval habitats of these two species have been poorly studied (Carpenter et al. 2008b). Pulicaris complex midges were encountered developing in small water-logged substrates near lakes (Kremer, 1965), bogs and small marshy places (Kettle and Lawson, 1952; Konurbayev, 1965), forest leaf-litter and ponds (Trukhan, 1975; Mirzaeva et al. 1976), proving their affinity to breed in wet soil and marshy areas (Tweddle, 2002). Only one specimen was captured by Uslu and Dik (2007) in moist soil with organic matter. A few *C. punctatus* and a single *C. pulicaris* emerged from a marsh polluted with organic material (Nielsen et al. 1998) and from wet grazing areas in Denmark (Kirkeby et al. 2009), despite the fact that large numbers of both species from light traps were found in the same substrate sampling places. In our study, light trap collections indicated that *C. lupicaris* was 40 times more abundant than *C. pulicaris*, assuming adults are equally attracted to light traps. There also was a 14-fold difference in individuals emerging from soil samples, indicating a high prevalence in the number of *C. lupicaris* compared with *C. pulicaris*. In northern Europe the presence of both species was recorded in several countries, but in contrast to our survey, *C. pulicaris* was the most common and widespread in light trap collections (Baldet et al. 2008; Casati et al. 2009; Vorsprach et al. 2009; Nielsen et al. 2010). Additionally, those two species were recovered in the majority of all sampled microhabitats (nine) in the present study, mainly in fallen leaves (35%), river edges (17%) and forest mud (16%). The remaining 32% emerged from other microhabitats (Table 4). Clearly these species are rather adaptable in choice of larval habitats. The sex ratio (♀/♂) in *C. lupicaris* was 1.08 for emerging midges, in contrast to light traps, which yielded 99.0% females. Even though literature usually has shown roughly equal sex ratios in reared *Culicoides* (Mullens and Rutz, 1983; Lysyk and Danyk, 2007) other authors have recorded skewed sex ratios from different *Culicoides* spp. (Kettle and Lawson, 1952; Braverman, 1978; Root and Gerhardt, 1991) and preponderance of females using vehicle-mounted traps (Sanders et al. 2012). According to our results, most species showed a slight proportional advantage in favour of females, except for *C. kibunensis* (Table 2).

It is possible that *C. punctatus* and *C. newsteadi* may play a role in BTV transmission as both are closely related to *C. pulicaris* and may have some susceptibility to BTV infection, but this remains to be confirmed (Mellor et al. 1981; Carpenter et al. 2006; Patakakis et al. 2009). Both species were also captured in large numbers (up to 321 specimens of *C. punctatus*

per sample) near the farm and in the pond shorelines (west and east). It did occur in the same pond places as its sibling species, *C. newsteadi*, but the latter species was relatively rare. Foxi and Delrio (2010) reported their presence mainly in the grassy edge of a pond associated with livestock.

6.4. Breeding sites of other species

Much more information is available on *C. impunctatus*, the most common midge in much of the U.K. It is particularly abundant along the west coast and in the Highlands of Scotland, where it far outnumbers all other *Culicoides* species (Bhasin et al. 2000). They live in a great variety of moist and wet terrestrial habitats, including marshes, swamp soils associated with *Myrica gale*, *Juncus* spp., *Sphagnum* spp., and peat-rich areas from Northern Europe (Kettle, 1990; Blackwell et al. 1994; Carpenter et al. 2001). Only two specimens were captured in our field light trapping, thus it seems to be very rare in Spain, appearing only occasionally in some farms (Ventura et al. 2005; Romón et al. 2012). Although only a very small fraction of this species appear to be capable of developing a fully disseminated BTV infection (Carpenter et al. 2006), it is important to consider for future studies.

The midges *C. festivipennis* and *C. circumscriptus* were very common species in the pond edge mud, and have been recorded as cosmopolitan and adaptable species capable of developing in almost all wet organic environments, particularly in substrates near pools of water, mud rich in organic matter, and moist soils (Uslu and Dik, 2010). In this study, both emerged especially from the west pond (*Juncus* spp.) and east pond (*Typha* spp.), living in permanently inundated soils near the waterline as indicated by Foxi and Delrio (2010).

Larval habitats of nine species, collected as adults, remain undiscovered, but almost all of them were rare or uncommon species (less than 100 specimens in light traps). The exception was *C. fagineus* (521 specimens). Its breeding sites are fairly well known, mostly in tree holes (Edwards et al. 1939; Dzhafarov, 1964; Kremer, 1965; Sánchez-Covisa et al. 1979). In previous studies we tried to set waxed paper cups with an adhesive layer over three holes (*Quercus* spp. and others), but no midges were captured. Uslu and Dik (2010) also did not collect *Culicoides* specimens in tree holes. Mellor and Pitzolis (1979) noted that scarce species were not encountered due to developing in very small numbers, or having a restricted distribution.

Curiously, no *Culicoides* species appeared in five places that seemed permissive for midge development: moss, manure dispersed as fertilizer in the pasture, pure sheep droppings inside the sheep-barn and predominantly muddy edge substrates from the river (Fig. 1). However, the manure dispersed over the pasture which was sampled exclusively at the end of the summer, which is the same that the heap of old-composted manure, could have produced *Culicoides* collections in other dates i.e. in the middle of the summer. Further studies are necessary to demonstrate more conclusively the absence of *Culicoides* species in such sites; we hypothesize there may be certain incompatibilities according to extremes values in physical parameters such as pH, dryness of the sample, and high soil compaction.

6.5. Vertical and horizontal distribution

The data regarding vertical distribution show larval preferences for the upper substrate layers, as mentioned by Kettle (1951), Lubega and Khamala (1976), Mullens and Rodriguez (1992), Blackwell and King (1997) and Uslu and Dik (2006). This is in contrast with Linley (1966), who observed the salt marsh species *C. furens* in deeper layers, ranging from 15 to 44 cm. In some species two main factors can influence larval movement: light effects (e.g. intensity or photoperiod) and water level fluctuation (Mullens and Rodriguez, 1988; Aussel and Linley, 1993). Different interpretations could be considered from previous studies, which reported several other factors such as type of soil, water temperature, presence of vegetation and distribution of predators as some probable parameters implicated in *Culicoides* larval distribution. Mullens and Rodriguez (1985) showed substantial and cyclical changes in *Culicoides sonorensis* larval numbers in polluted surface shoreline mud during different times of day, and larvae responded positively to light versus shading treatments. We hypothesize that diel variation in oxygen availability could be involved (gas diffusion through the cuticle is needed for these apneustic insects), with higher numbers in shoreline surface mud at hotter midday periods, when oxygen levels could be too low for larval survival in deeper mud or under water. So, larvae of other *Culicoides* species may be capable of substantial movement in their habitats even over short periods.

In the present study, samples of fresh manure were totally dried on the surface, but larvae were recovered at deeper layers, where higher levels of moisture remained. Larvae of *C. sonorensis* (as *C. variipennis*) normally require standing water, but some larvae can survive drying mud conditions (18-25% moisture) at depths of 3-10 cm for periods of at least 5-7 days

(Mullens and Rodriguez, 1992). Similar conditions probably occurred in the muddy margins of the pond in the present study, where the top 1-3 cm were extremely dry.

The horizontal distribution of larvae near the pond could be explained according to the previously mentioned factors (water level) surrounding the pond. Presumably larval survival was better in those places with high levels of moisture, closer to the waterline.

6.6. Seasonal collections

Midges were present in outdoor light traps from May onwards while the numbers collected increased sharply in July. In soil samples, the maximum peak of emergence overall occurred in June, decreasing over the following months. Some temporal asynchrony was observed, but the majority of the emergence was from manure samples and for that, it would be desirable to take a larger number of samples (perhaps more frequently) for future studies. In both cases only a few specimens were recorded in March and November, and none in the intervening winter months. The severe winters in the northeast part of Alava, with hard frost periods between January and February, seemed to prevent the flight of *Culicoides* midges in the coldest months. In Turkey, *Culicoides* larvae and pupae were found exclusively between April and October, with the maximum number in August (high humidity and temperature) (Uslu and Dik, 2005, 2011). These authors also mentioned that the lower temperatures during winter months in Konya could be responsible for the absence of *Culicoides* larvae and pupae in that location. A critical question is: where and in what stage(s) do *Culicoides* species live during the winter? Four main and nonexclusive hypotheses are: (i) they pass the winter as larvae, perhaps in unknown or very specific places (ii) eggs overwinter and hatch from March onward (iii) adults survive resting inside barn installations and (iv) *Culicoides* midges arrive from other (perhaps warmer) regions.

In addition, in October and November a low peak of adult activity was recorded in the light traps. This was mostly due to the bivoltine pattern of *C. lupicaris*, with a second generation in the middle of autumn. Similar results were reported by Foxi et al. (2011) with *C. pulicaris* collections at the end of the autumn. However, this peak was insignificant in emergence traps and there principally was due to emergence of smaller numbers of *C. obsoletus/C. scoticus* species, rather than *C. lupicaris*. A similar situation occurred with *C. achrayi* and *C. furcillatus*, with an absence of emergence from collections in soil samples, in contrast to some continuing adult activity evident from light traps. This could be due to (i) the

existence of other breeding sites, not considered in this study (ii) the high potential attraction of the UV-light, which could draw *Culicoides* midges from long distances and (iii) the modification of the initial conditions (light, confinement, humidity gradient) culminating in abortive larval/pupal development in our breeding habitat collections. Unfortunately, the use of emergence traps in the field is currently restricted owing to human vandalism and livestock trampling. Even so, we suggest for future research the use of bigger and durable emergence traps placed in the field to compare the yield with emergence traps holding field-collected samples in the laboratory.

7. CONCLUSIONS

Culicoides obsoletus has a strong association with livestock, and the vast majority of them were developing in different types of manure or manure-laden soil substrates. In contrast, the sibling species *C. scoticus* seemed to be more dispersed and less common, preferring to occupy forest habitats with leaf litter. The Pulicaris group has been found in a great variety of environments, particularly breeding near areas of standing water. As has been demonstrated, manure is the principal breeding site of *C. obsoletus* s.s. However, chemical signals involved in breeding site location remain unclear and almost completely uninvestigated. To determine which chemicals are capable of eliciting a positive flight response towards manure (or perhaps other breeding media), additional analytical and biological studies would be needed, i.e. headspace volatile collection and behavioural bioassays (electroantennogram techniques and multiple-choice assays). Proper manure management, perhaps coupled with extensive trapping using odour-attractants sufficiently potent or/and insecticide-treated targets for *Culicoides* midges in the framework of a “push-pull” strategy, could reduce the midge populations in localized or isolated areas and therefore reduce the possibility of arbovirus transmission. Owing to the geographic position of Spain and evidence of progressive climate change, it is likely that more arboviruses could reach those latitudes. In that case, biting midges (and other vectors such as mosquitoes) could have an increasing role in pathogen transmission.

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CHAPTER 4

Version of the chapter 4 based on the following publication:

Laboratory and field evaluation of chemical and plant-derived potential repellents against *Culicoides* midges (Diptera: Ceratopogonidae) in the Basque Country, Northern Spain (Submitted to Veterinary Parasitology)

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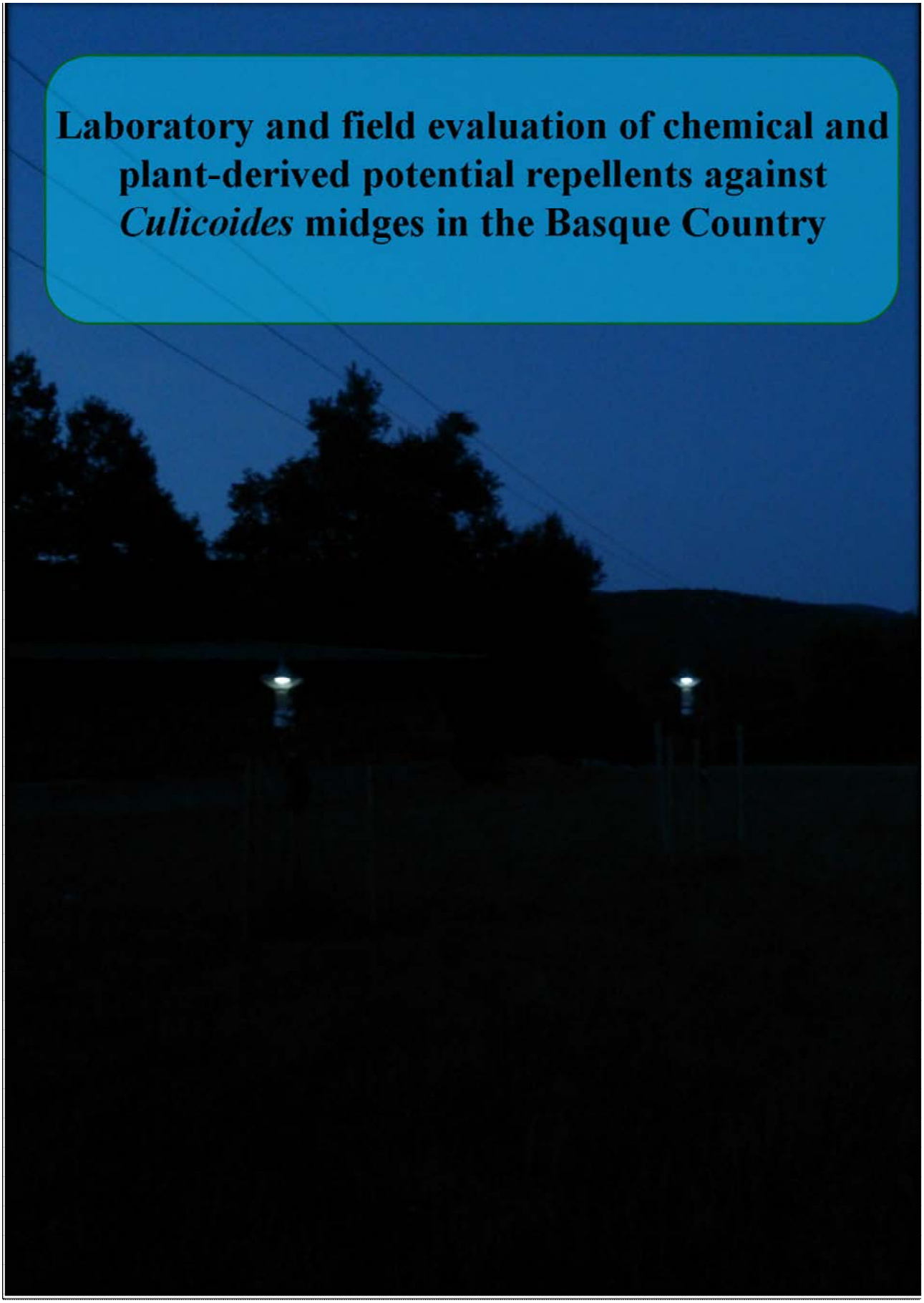
Laboratory and field evaluation of chemical and plant-derived potential repellents against *Culicoides* midges in the Basque Country

LABORATORY AND FIELD EVALUATION OF CHEMICAL AND PLANT-DERIVED POTENTIAL REPELLENTS AGAINST *CULICOIDES* MIDGES IN THE BASQUE COUNTRY

1. ABSTRACT

The efficacy of 23 compounds in repelling *Culicoides* biting midges, particularly *Culicoides obsoletus* females, was determined by means of a Y-tube olfactometer. The ten most effective were further evaluated in landing-bioassays. The six most promising (including chemical and plant-derived repellents) were evaluated at 10% and 25% concentrations in field assays using CDC-light traps. At least three compounds showed promising results against *Culicoides* biting midges with the methodologies used. While olfactometer assays indicated DEET at 1 μg/μl to be the most effective, landing-bioassays showed plant derived oils to be better. Light traps fitted with polyester mesh impregnated with a mixture of octanoic, decanoic and nonanoic fatty acids at 10% and 25% concentrations collected 2.2 and 3.6 times fewer midges than control traps and were as effective as DEET, presently considered the gold standard in insect repellence. The best plant-derived product was lemon eucalyptus oil. Although these have been reported as safe potential repellents the present results indicate DEET and the mixture of organic fatty acids to be superior and also longer lasting.

2. KEY WORDS Synthetic repellents, Plant extracts repellents, *Culicoides obsoletus*, Y-tube olfactometer, Landing-assays, Light traps

3. INTRODUCTION

Culicoides biting midges are an important genus with regard to the health of livestock. Notwithstanding the fact that midges are a severe biting nuisance in some parts of the world certain members of this Genus were shown to be involved in the transmission of at least 66 different viruses, 15 protozoans and 26 filarial worms worldwide (Meiswinkel et al. 2004; Borkent, 2005). The veterinary importance of these midges in Spain was highlighted when bluetongue virus (BTV), a devastating orbiviral disease affecting ruminants and transmitted by *Culicoides* midges, entered the Balearic islands in 2000 and subsequently spread across almost the whole Iberian Peninsula during 2004 and 2008

(Pérez de Diego et al. 2013). After the first detection of BTV in northwest Europe in 2006 the virus spread rapidly throughout northern Europe (Wilson and Mellor, 2009). In 2011 a previously unknown *orthobunyavirus*, Schmallenberg virus (SBV) also apparently transmitted by *Culicoides* midges (Veronesi et al. 2013), was detected in Germany. It caused severe economic loses for sheep and cattle breeders in Europe (Hoffmman et al. 2012).

Of the 81 species of *Culicoides* recorded in Spain (Alarcón-Elbal and Lucientes, 2012), *Culicoides obsoletus* are considered the most abundant livestock associated species in northern Spain (Romón et al. 2012; González et al. 2013a). They are found widely distributed throughout the Western Palaearctic region (Carpenter et al. 2008) and are considered a likely potential vector of BTV and SBV (Wilson and Mellor, 2009; Veronesi et al. 2013).

The effective control and monitoring of *Culicoides* midges is hampered by their small size (<3 mm), predominantly nocturnal activity and the wide, and mostly unknown, range of microhabitats that can be utilized as possible breeding sites (González et al. 2013b; Harrup et al. 2013). These factors could have contributed to the failure of the large scale use of insecticides to control midges in the past (Satta et al. 2004).

The effective use of repellents to reduce the biting rate of *Culicoides* midges could contribute to the more effective integrated control of the pathogens transmitted by them. This approach could support current control strategies and may be particularly relevant where appropriate vaccines are not available e.g. for SBV or during outbreak situations (Mellor and Hamblin, 2004).

Available insect repellents can be classified as either synthetic or plant-derived. Amongst the synthetic repellents, DEET (*N,N*-Diethyl-3-methylbenzamide) is considered to be one of the most efficient insect repellents worldwide (Katz et al. 2008). Although the activity of DEET in mosquitoes and other insects is widely documented, information regarding its effectiveness against *Culicoides* midges is limited to a few studies focusing mainly on the afrotropical *C. imicola* and North American *C. sonorensis* (Braverman et al. 1997; Braverman and Chizov-Ginzburg, 1998; Page et al. 2009). Preliminary studies in the Basque Country showed significant repellence effects of DEET against *C. obsoletus/C. scoticus, C. lupicaris* and *C. achrayi* (Romón et al. unpublished).

Plant-derived repellents consist of a wide range of natural compounds that can act as fumigants, contact insecticides or anti-feedants. Of thousands of plant-derived essential oils that have been evaluated as potential repellents, relatively few have demonstrated the broad effectiveness and long-lasting protection of DEET (Fradin, 1998). Some essential oils, e.g. neem oil, lemon eucalyptus oil and extracts derived from Meliaceae and Myricaceae have, however, demonstrated repellent effects in some *Culicoides* species in Scotland (Blackwell et al. 2004).

Despite the apparent efficiency and widespread use of DEET, there are still some unresolved controversies concerning the safety and mechanisms of action (Corbel et al. 2009). In addition, it was shown that *Aedes aegypti* can become desenthetized following exposure and that there could be a decrease in effectiveness of DEET (Stanczyk et al. 2013).

The continuous development and evaluation of synthetic/chemical formulations to replace or complement DEET-based repellents will be essential for the integrated control of the pathogens transmitted by insect vectors. Due to the medical importance of mosquitoes most studies involving repellents are skewed towards their applicability to mosquitoes. Based on their veterinary importance it will be necessary to expand these studies to include *Culicoides* midges.

In the present study the efficacy of 23 repellents, previously showing successful effects in congeneric and/or closely related mosquitoes, were evaluated in the laboratory and field for their effectiveness against *Culicoides* species.

4. MATERIAL AND METHODS

4.1. Compounds evaluated

The category, chemical composition and/or formula and purity of 23 compounds, previously shown to repel either mosquitoes or *Culicoides* midges, are shown in Table 1. Of the compounds evaluated 12 were of chemical or synthetic origin while 11 were plant or botanical (Table 1). The potential synergistic effect of four mixtures of these compounds was also determined (Table 1). All compounds, except Picaridin/BayRepel, (KBR3023) and IR3535 which were obtained from commercial chemists, were purchased from Sigma-

Aldrich (Madrid, Spain). After screening with Y-tube olfactometer and landing-assays the most promising compounds were evaluated under field conditions with light traps.

Table 1. Compounds and/or mixtures evaluated against *Culicoides obsoletus* in laboratory assays (2-choice olfactometer and landing-bioassays)

N	Commercial name	Category	Composition/Formula	Purity
1	Cypermethrin	Pyrethroid	$C_{22}H_{19}C_{l2}NO_3$	99%
2	DEET	Organochloride	*N*,*N*-Diethyl-3-methylbenzamide	≥97%
3	Geranyl acetone	Syntethic	[(E)-6,10-Dimethyl-5,9-undecadien-2-one], mixture isomers	≥97%
4	Picaridin/BayRepel (KBR3023)	Syntethic	1-Piperidine carboxilic acid	50%
5	IR3535	Syntethic	3-[N-butyl-N-acetyl] aminopropionic acid ethyl ester	50%
6	Decanal	Aldehyde	$CH_3(CH_2)_8CHO$	95%
7	Nonanal	Aldehyde	$CH_3(CH_2)_7CHO$	95%
8	Octanal	Aldehyde	$CH_3(CH_2)_6CHO$	99%
9	6-methyl-5-hepten-2-one	Natural	$(CH_3)2C=CHCH_2CH_2COCH_3$	≥98%
10	Neem (Azadirachtin) oil	Plant derived	*Azadirachta indica* oil	High
11	Lavander oil	Plant derived	*Lavandula angustifolia* oil	High
12	Rhodinol (lemon grass) oil	Plant derived	*Cymbopogon citratus* ct *rhodinol* (Mixture of L-citronellol and geraniol)	High
13	Lemon eucalyptus oil	Plant derived	*Eucalyptus maculata* var. *citriodora* oil (PMD)	High
14	Rosemary oil	Plant derived	*Rosmarinus officinalis* oil	High
15	Jasmine oil	Plant derived	*Jasminum grandiflorum* oil (mix eugenol 2.1%, farnesol 0.1%, geraniol 0.1% and linalol 4%)	High
16	Balm leaves oil	Plant derived	*Melissa officinalis* oil (citral, citronellol, geraniol and linalool)	High
17	Lemon oil	Plant derived	*Citrus limon* oil (citronellol)	High
18	Limonene	Terpene	$C_{10}H_{16}$ (sum of enantiomers)	90%
19	4-Propylphenol	Organic compound	$C_9H_{12}O$	99%
Mix 1	Mix: 1:1:1 (jasmine + lavander + rosemary)	Plant derived	Mixture of natural plant extracts	High
Mix 2	Mix: 1:1 (geranyl acetone + 6-methyl-5-hepten-2-one)	Mixture	Mixture of human odour constituents	High
Mix 3	Mix: 1:1:1 (octanoic, nonanoic + decanoic)	Acids	Mixture of fatty acids (C8, C9, C10)	96-98%
Mix 4	Mix: 1:1:1:1 (Balm + Rhodinol + lemon eucalyptus + lemon oil)	Plant derived	Mixture of four components with citronellol as constituent	High

4.2. Origin and collection of test insects

For olfactometer assays freshly emerged nulliparous *C. obsoletus* females were obtained from old-manure heaps on a farm near Barrundia, Elguea (42º55´59´´N; 02º30´51´´E; 754 m above sea level) in the north of Alava province (Basque Country) northern Spain as previously described (Foxi and Delrio, 2010) (Fig. 1). Previous studies

indicated that more than 99% of the specimens emerging from these manure heaps to be *C. obsoletus* (González et al., 2013b). After emergence in the laboratory *C. obsoletus* were aspirated and transferred to acclimatization cages and maintained at 22-26ºC and 50-60% RH on a 15% sucrose solution provided on cotton wool pledgets until used. For landing-bioassays *C. obsoletus* females were collected alive with suction light traps (CDC blacklight 4W model 1212, Entomopraxis, Barcelona, Spain) near sheep (Fig. 2).

The collected females were anesthetized with CO_2 and identified to species level prior the Y-tube olfactometer and landing behaviour assays.

Fig. 1. Rearing mosquito cages with funnel emergence devices.

Fig. 2. CDC-UV-light trap showing the modification to collect alive specimens.

4.3. Y-tube olfactometer assays

Repellence responses of *C. obsoletus* females were determined using a Y-tube olfactometer (ARS, Analytical Research system, Micanopy, Florida, USA) and it was adapted to the smaller size of the midges. The main tube was 150 mm long and 20 mm in diameter. The arms were 100 mm long and 15 mm in diameter with a 90º angle between them. Regulated air flow was maintained at 0.3 L/min with a filtered charcoal-humidified air-stream connected to the two glass chambers containing the test compound located at the proximal ends of the short arms. The olfactometer was installed in a light-tight box. To instigate movement in the insects a cold light source was placed equidistant from each of the arms (Blackwell et al. 1994) (Fig. 3A).

Each of the 23 compounds was evaluated at 1, 0.1 and 0.01 µg/µl dilutions in hexane. Ten microlitres of the relevant compound was placed on Whatman filter papers (10 mm diameter) and air dried for 30 seconds before inserting it into one of the short arms of the olfactometer. An identical paper with pure hexane was used as a control in the second arm.

For each replicate 10-12 insects were immobilised at -4°C for 30 seconds before inserting them into the main tube of the olfactometer. Ten replicates of each component were done. To avoid directional bias the arms were reversed after five replicates. The behavioural responses of the midges were observed after 4 min: positive (midges which advanced at least 4 cm forward into one of the arms) and negative (uplight orientation and/or inactivation) (Fig. 3B).

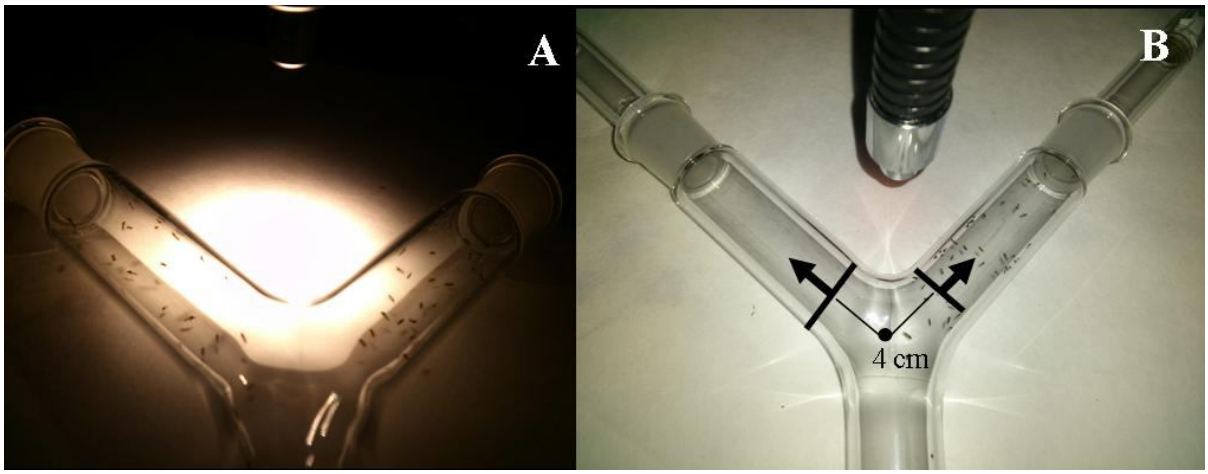

Fig. 3. Y-tube olfactometer **(A)** Cold light source infraring on the middle of the short arms of olfactemeter **(B)** Regions of the olfactometer; positive responses are considered when midges are inside the region above black lines (4 cm from the angle between short arms).

Assays were conducted from May to July 2012. Tests were done between 7.00 a.m. and 10.00 a.m. and each insect was tested only once until a maximum of three days after emergence.

After 4 minutes the percentage repellence was calculated as: 100 x N/T; where N = number of *Culicoides* repelled (i.e. the number of midges which advanced at least 4 cm into the arm containing the control) and T = number of midges which advanced at least 4 cm forward into either one of the two arms.

2.4. Landing-bioassays

The ten most effective repellents, determined in the olfactometer, were further evaluated in landing-bioassays. For this purpose six conical opaque plastic tubes 150 mm in length and 50 mm diameter were inserted 150 mm apart into one of the vertical sides of a gauze mosquito cage (600 mm x 600 mm x 600 mm). To initiate movement towards these tubes the mosquito cage was lined with black paper and the tubes homogeneously illuminated by infrared light (Fig. 4).

Circular Whatman filter papers with the same diameter as the tubes were impregnated with 100 µl of the relevant solution at a concentration 1 µg/µl. After 30 seconds of air drying these papers were attached to the conical outlets of three of the six tubes. The remaining three tubes served as controls and were loaded with filter papers impregnated with 100 µl hexane. The position of the six tubes was alternated in six replicates in a randomized Latin square design. Freshly impregnated filter papers were used for each replicate. Batches of 20 to 30 anesthetized *C. obsoletus* females were released into the cage opposite the tubes. The number of midges on the filters papers was counted 2 seconds and 5 minutes after release. A fresh batch of midges was used in each trial and to avoid residual air contamination only one repellent was evaluated per day. Percentage of repellence for each time period was calculated as: 100 x N/T; where N = total number of *Culicoides* sitting on the three control papers and T = total number of *Culicoides* on all six filter papers (control + test). Assays were conducted in August 2012.

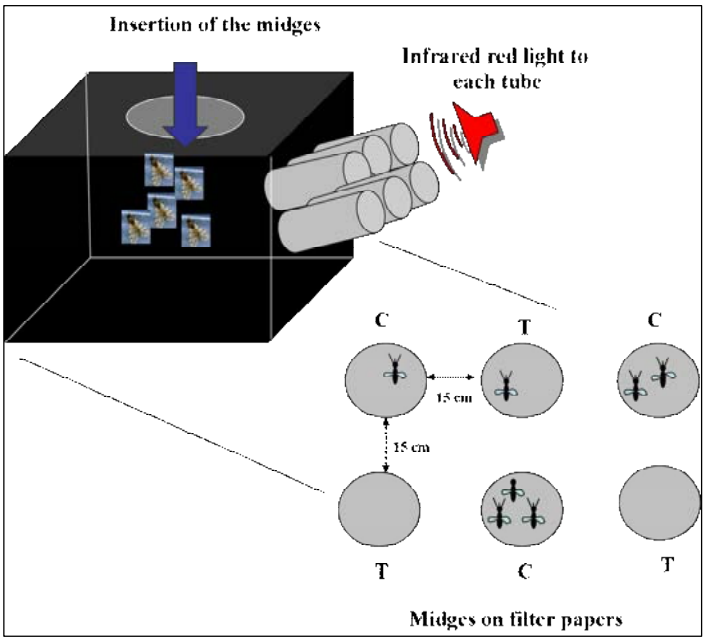

Fig. 4. Drawing of landing-bioassays. Female midges are inserted through a top window and rapidly are attracted to one of the six infrared tubes (3 control vs 3 treated).

2.5. Field trap evaluation

2.5.1. Compounds tested and test procedure

The chemical compounds evaluated with light traps under field conditions were (1) a 1:1 mixture of geranyl acetone and 6-methyl-5-hepten-2-one, (Mix 2), (2) a 1:1:1 mixture of octanoic, nonanoic and decanoic fatty acids, (Mix 3), (3) DEET the gold standard and (4) a control (99% ethanol) (Tables 1-3). The chemicals were evaluated at 10% and 25% concentrations. Trapping was conducted using CDC white light traps for 24 nights in the first part of summer 2013. Each concentration was evaluated in three replicates (12 nights) of a 4 x 4 randomised Latin square design (Page et al. 2009; Venter et al. 2011).

Plant-extracts evaluated were: (1) lemon eucalyptus oil, (2) a 1:1:1 mixture of jasmine, lavender and rosemary oil, (Mix 1), (3) lavender oil, (4) DEET and (5) a control (99% ethanol) (Tables 1-4). The extracts were evaluated at 10 and 25% concentrations for 20 nights at the end of summer 2013. Each concentration was evaluated in two replicates of a 5 x 5 randomised Latin square design. Collections from nights with adverse wether conditions or trap failure were repeated the following night.

The traps were located 12 meters apart in front of a stable housing approximately 150 sheep (Fig. 5). An hour before sunset polyester mesh (surface area 0.15 m^2, mesh size 2 mm) was immersed for 30 minutes in the relevant test solution. They were air-dried for 30 minutes before being attached to the traps with Velcro strips (Fig. 6). The amount of test preparation absorbed by the meshes after 30 min of air drying was calculated to ensure that polyester were suitable for repellents absorption.

An automatic time switch ensured that traps were operational for five hours after sunset. Midges were collected into water to which a drop of odourless detergent was added to break the surface tension.

The catches were retrieved each morning and the mesh discarded. All *Culicoides* midges were counted, sexed and identified to species level using appropriate keys (Delécolle, 1985; González and Goldarazena, 2011). The males of all species were grouped.

Fig. 5. Row of light traps lined in the meadow.

Fig. 6. White light CDC trap with polyester mesh at dusk.

The catches were retrieved each morning and the meshes discarded. All *Culicoides* midges were counted, sexed and identified to species level using appropriate keys (Delécolle, 1985; González and Goldarazena, 2011). The males of all species were grouped.

2.6. Statistical analysis

Chi-square (χ^2) testing was conducted to determine if the number of *C. obsoletus* in the two arm of the olfactometer were significantly (α = 0.05) different from the expected 50:50 distribution in the absence of any stimuli.

Statistical analyses of field trials were carried out with SPSS (2004) and significance level was defined as $P \leq 0.05$. If the data were not normally distributed, square root transformations were performed prior to conducting statistical analyses. If zero counts were observed, all data were (1 + x) transformed. Analysis of variance (ANOVA) was used to differentiate between the trap treatment effects; means were compared using Fisher protected *t*-test least significant difference (LSD).

5. RESULTS

5.1. Olfactometer

Three concentrations of 23 compounds were evaluated with the Y-tube olfactometer (Table 2). Relatively high numbers of midges responded positively towards one of the olfactometer arms. Except for cypermethrin (50.4%), mixture 3 (62.8%) and nonanal (66.7%) this positive response was >70% for all compounds (Table 2).

The percentage of repellence for *C. obsoletus* ranged between 97.8% (DEET) to 45.4% (neem oil) at 1 µg/µl, from 91.3% (4-propyl-phenol) to 36.8% (KBR3023) at 0.1 µg/µl and from 86.6% (jasmine) to 45.8% (rosemary) at 0.01 µg/µl (Table 2). At 1 µg/µl concentrations, with the exception of limonene and neem, statistically more midges were found in the control arm than the arm containing the potential repellent (Table 2). At 0.1 µg/µl concentrations only 12 of these compounds show a significant repellence and at 0.01 µg/µl this number falls to eight (Table 2). With the exception of DEET, essential oils exhibited a higher degree of repellence than the chemical compounds.

DEET elicited the highest efficacy at 1 µg/µl (97.8%), 4-propyl-phenol at 0.1 µg/µl (91.3%) and jasmine at 0.01 µg/µl (86.3%) (Table 2). In general, relatively high percentages of repellence were observed with plant-derived oils such as jasmine (93.9%), lavender oil (93.7%), and a mixture of these two with rosemary oil (mix 1) (94.4%) at 1 µg/µl (Table 2). Likewise, the mixture of fatty acids (mix 3) gave relatively high repellence (Table 2). In contrast, neem oil and limonene had low repellent properties against *C. obsoletus* at all doses (Table 2).

Table 2. Percentage repellence obtained with 19 single repellents and four mixtures, at 1 µg/µl, 0.1µg/µl, 0.01 µg/µl, against *Culicoides obsoletus* as determined with a Y-tube olfactometer.

Compound	1µg			0.1µg		0.01µg	
	+	C	P-value	C	P-value	C	P-value
DEET	76.0	97.8	0.000	86.0	0.000	66.0	0.0237
Mixture 1	85.7	94.4	0.000	78.1	0.000	71.4	0.0013
Jasmine	90.0	93.9	0.000	86.6	0.000	86.4	0.0003
Lavender	87.2	93.7	0.000	74.3	0.000	61.4	0.0558
4-propyl-phenol	92.7	91.0	0.000	91.3	0.000	57.2	0.1271
Eucalyptus oil	81.5	90.5	0.000	48.1	0.785	66.7	0.0892
Mixture 3	62.8	89.5	0.000	73.8	0.002	55.0	0.5271
Balm leaves	87.1	88.4	0.000	80.4	0.000	65.9	0.0348
Mixture 2	78.9	87.5	0.000	76.3	0.001	71.7	0.0002
Mixture 4	74.2	85.7	0.000	80.7	0.000	66.6	0.0068
Rhodinol	81.7	85.7	0.000	86.9	0.000	68.0	0.0109
Geranyl acetone	80.6	85.0	0.000	76.3	0.001	78.6	0.0002
Decanal	85.6	82.6	0.000	67.5	0.003	57.1	0.3545
Octanal	90.8	81.6	0.000	54.9	0.414	52.5	0.6547
Nonanal	66.7	81.6	0.000	46.7	0.460	54.5	0.4902
IR3535	84.3	80.8	0.000	58.3	0.157	52.7	0.7389
Lemon oil	84.8	72.7	0.000	62.5	0.083	53.1	0.6171
KBR3023	76.7	71.4	0.005	36.8	0.104	53.8	0.5791
Rosemary	89.5	70.0	0.001	53.4	0.550	45.8	0.4795
Cypermethrin	50.4	66.7	0.020	50.0	1.000	69.3	0.0114
6-methyl-5-hepten-2-one	88.2	66.6	0.006	60.8	0.140	52.2	0.2528
Limonene	91.5	57.9	0.135	56.2	0.317	48.4	0.7995
Neem (Azadirachtin)	81.4	45.5	0.460	52.7	0.637	50.0	1.0000

Results are expressed as percentage of midges found in the control arm (C) calculated from choice responses (+). P value was obtained with Chi-square test (α= 0.05). N=100-120 specimens per treatment.

5.2. Landing-bioassays

The percentage repellence after 2 seconds and 5 minutes for each of the ten compounds are depicted in Figure 7. For all ten compounds significantly more midges were found on the control than on papers containing the potential repellent (Fig. 7). The compound with the highest repellent activity at 2 seconds after release was lemon eucalyptus. At 2 seconds 100% of the midges were found on the control and none on the paper with lemon eucalyptus (Fig. 7). Lemon eucalyptus was followed by lavender (96.1%), mix 4 (92-94%)

and jasmine (90.0%) (Fig. 7). Surprisingly DEET only elicited 76.1% repellence and mixtures 1 and 3 (the best in the olfactometer) gave less than 75% repellence (Fig. 7).

Five minutes after release 100% repellence was found for lemon eucalyptus, lavender, mix 4, jasmine, 4-propyl-phenol and balm leaves (Fig. 7). For DEET, mix 1, mix 2 and mix 3 the number of midges on the control papers did not increase from that observed after 2 seconds (Fig. 7).

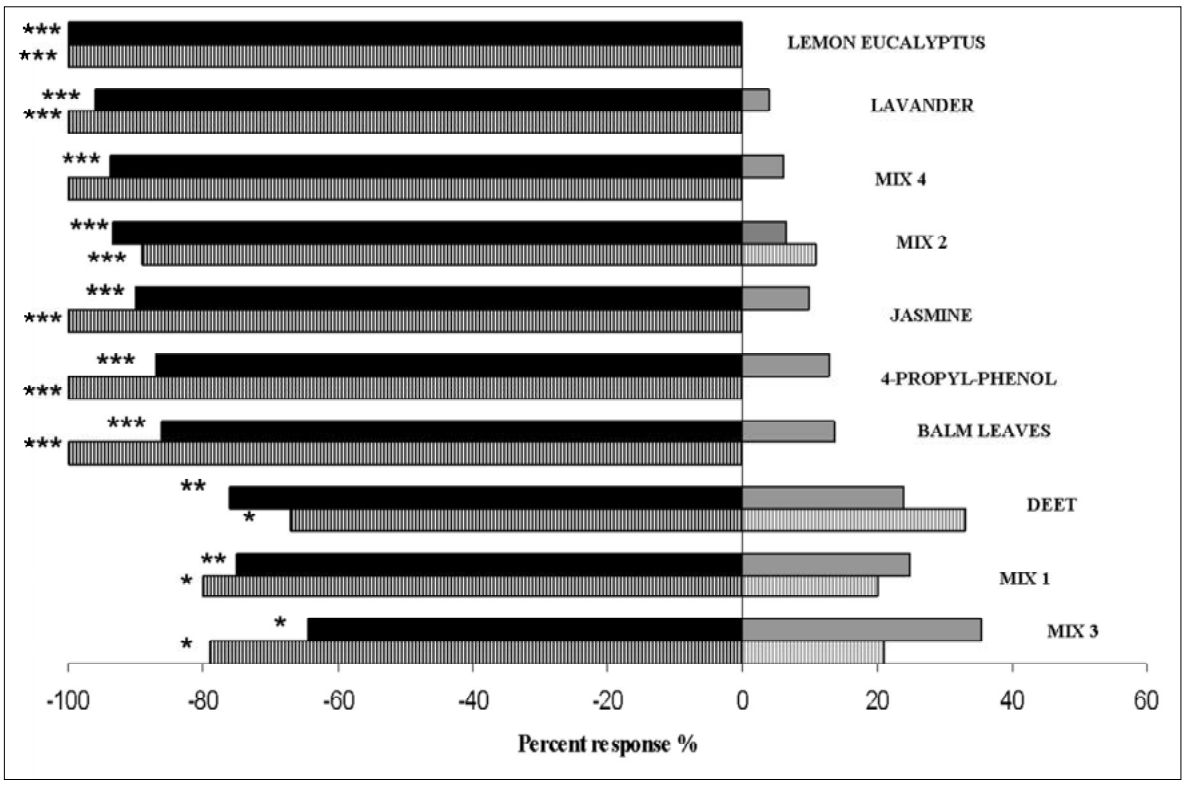

Fig. 7. *Culicoides obsoletus* exposed to spatial repellent chemicals within a mosquito cage at doses (1µg/µl). The results are expressed as percentage of the number of midges that alighted on filter papers after 2 seconds (blackish-greyish) or after 5 min (hatched bars). N= 20 specimens per replicate (6 replicates per repellent). Compounds are ordered by powerful repellency effect of the first trial. Mix 4: Rhodinol + lemon eucalyptus + lemon oil; Mix 2: geranyl acetone + 6-methyl-5-hepten-2-one; Mix 1: jasmine + lavander + rosemary; Mix 3: octanoic + nonanoic + decanoic acids. Asterisk denote significance using Chi-square test (* P<0.05; ** P< 0.01; *** P<0.001).

5.3. Light trap evaluation

The number of midges and species breakdown of the 48 collection made at 10% and 25% concentration of the chemicals are shown in Table 3. At both concentrations fewer midges were collected in the three baited traps than with the control (Table 3). At the 10% level the mean number of midges collected with the mixture of fatty acids (Mix 3) (285.5 ±408.6) was significantly (P = 0.030) lower than with the control (641.8 ±872.4) or the trap with Mix 2 (459.7 ±588.1) (Table 3). The lower mean numbers collected with Mix 2 and

DEET (313.8 ±385.5) were not significantly different from the control (Table 3). Also at 25% concentration the lower mean with Mix 3 (198.8 ±125.5) and DEET (298.2 ±144.2) was significantly (P = 0.000) lower than the control (716.8 ±223.6) (Table 3). At this higher concentration the mean number collected with the DEET trap was significantly (P = 0.000) lower than with Mix 2 (581.8 ±381.7) (Table 3).

While 18 *Culicoides* species were collected in the control trap at the 10% concentration only 13 species each were collected with DEET and Mix 3 (Table 3). The dominant species, ranging from 49.1% (control) to 33.7% (DEET) in all four treatments were *C. brunnicans* (Table 3). The proportional representation of *C. brunnicans* differs significantly between treatments (X^2 = 270.92, df = 3, P < 0.001). Like the total mean numbers the three treated trap collected fewer *C. brunnicans* than the controls (Table 3). Also for *C. brunnicans* only the numbers collected with the Mix 3 (122.0 ±213.3) was significantly (P = 0.021) less than that of the control (315.4 ±509.7) (Table 3). Similar results were obtained for *C. obsoletus* and *C. scoticus* (grouped) and the subgenus *Oecacta* (Table 3). For other species and the males the numbers collected, although lower, were not significantly different from the control (Table 3). A further six species at <4% were not analysed further.

In the 48 collection made of the 25% concentration 20 species were collected with the control and 16 each with the Mix 3 and DEET traps (Table 3). *Culicoides lupicaris* and *C. pulicaris*, as a group, replaced *C. brunnicans* as the dominant grouping (Table 3). Except for the *C. obsoletus/C. scoticus* grouping and the subgenus *Silvaticulicoides* and *Oecacta*, the mean numbers of the different species collected with the DEET and Mix 3 traps, although not statistically different from each other, were lower than the control trap (Table 3). For the *C. obsoleus/C. scoticus* grouping significantly (P = 0.000) lower numbers were collected with Mix 3 (24.7 ±17.1) than with DEET (56.5 ±30.3) (Table 3). For the subgenus *Silvaticulicoides* the numbers collected were not significantly different (P = 0.000) between treatments (Table 3).

The number of midges and species breakdown of 50 collections at 10% and 25% concentration of plant repellents are shown in Table 4. At 10%, significantly (P = 0.002) fewer midges were collected in the four treated traps compared to the control (112.1 ±48.6) (Table 4). At 25% the lower mean number of *Culicoides* midges collected with mix 1 (32.8

±24.7) and Lavender (49.9 ±41.1) was not statistically different from the control (60.0 ±43.6) (Table 4).

At the 10% level 16 species were collected in the control trap whereas DEET and Eucalyptus captured 12 species (Table 4). The dominant species collected, ranging from 36.7% (control) to 23.7% (Lavender) were *C. lupicaris* (Table 4). The proportional representation differs significantly between treatments (X^2= 41.524, df = 4, $P < 0.001$). Similar to the mean numbers significantly ($P = 0.008$) fewer midges were collected in the treated traps than in the control (Table 4). Also members of the *C. obsoletus*/*C. scoticus* ($P = 0.004$) and the subgenus *Oecacta* ($P = 0.026$) were collected in significantly lower numbers in the treated than the control traps (Table 4). In addition the members of *C. obsoletus*/*C. scoticus* grouping were collected in significantly lower numbers with DEET (27.0. ±13.9) than with Eucalyptus (18.2 ±6.0) (Table 4).

Due to the prolonged drought, the numbers and species collected at the 25% concentration were lower than in the previous evaluations (Table 4). Although *C. lupicaris* was still the dominant species in the control trap and those with Lavender, members of the *C. obsoletus*/*C. scoticus* were the dominant grouping in the other three traps (Table 4). The mean numbers of *C. lupicaris* collected in the control (24.5 ±26.5) was significantly higher than with DEET (6.5 ±5.9), Eucalyptus (7.1 ±5.4) and Mix 1 (9.5 ±8.6) ($P = 0.002$) (Table 4). The lower mean numbers of the *C. obsoletus*/*C. scoticus* grouping collected with DEET (8.2 ±12.1) was significantly ($P = 0.038$) lower than Mix 1, lavender and the control (Table 4).

Table 3. *Culicoides* midges collected with four traps impregnated with three different chemical treatments at 10% and 25% (w/w) during summer of 2013 at a sheep farm in the Basque Country region, Spain. 12 collections were made with each repellent.

		10% (w/w)					**25% (w/w)**				
Species	Treatment	DEET	Mix 2	Mix 3	Control	P value	DEET	Mix 2	Mix 3	Control	P value
No. of species collected			17	13	18		16	19	16	20	
Total *Culicoides* collected (%)		3 766 (18.4)**ab**	5 516 (27.0)**a**	3 426 (16.8)**b**	7 702 (37.7)**a**	0.030	3 578 (16.6)**b**	6 982 (32.4)**a**	2 386 (11.1)**b**	8 601 (39.9) **a**	0.000
Mean collected (±SD)		313.8 (385.8)	459.7 (588.1)	285.5 (408.6)	641.8 (872.4)		298.2 (144.2)	581.8 (381.7)	198.8 (125.5)	716.8 (223.6)	
C. brunnicans	Total collected	1271 (13.8)**ab**	2638 (28.8)**a**	1464 (16.0)**b**	3785 (41.3) **a**	0.021	73 (8.3)**b**	278 (31.9) **a**	78 (8.9)**b**	442 (50.7)**a**	0.000
	Mean collected (±SD)	106.0 (141.7)	219.8 (342.6)	122.0 (2313.3)	315.4 (509.7)		6.1 (5.5)	23.2 (20.3)	6.5 (6.4)	36.8 (30.8)	
	% total of *Culicoides*	33.7	47.8	42.7	49.1		2.0	4.0	3.3	5.1	
C. punctatus	Total collected	978 (22.3)	1138 (25.9)	757 (17.2)	1511 (34.4)	0.382	308 (16.1)**b**	624 (32.7) **a**	217 (11.3)**b**	760 (40.0)**a**	0.000
	Mean collected (±SD)	81.5 (111.8)	94.8 (126.4)	63.1 (95.1)	125.9 (174.0)		25.7 (90.5)	52.0 (28.8)	18.1 (41.5)	63.3 (26.5)	
	% total of *Culicoides*	26.0	20.6	22.1	19.6		8.6	8.9	9.1	8.8	
C. lupicaris + *C. pulicaris*	Total collected	295 (28.7)	187 (18.2)	164 (16.0)	380 (37.0)	0.324	1245 (16.0)**b**	2690 (34.7)**a**	896 (11.5)**b**	2928 (37.7)**a**	0.004
	Mean collected (±SD)	24.6 (39.6)	15.6 (14.2)	13.7 (17.1)	31.7 (39.9)		103.8 (87.3)	224.2 (203.7)	74.7 (74.1)	244.0 (170.3)	
	% total of *Culicoides*	7.8	3.4	4.8	4.9		34.8	38.5	37.6	34.0	
C. obsoletus + *C. scoticus*	Total collected	144 (21.4)**ab**	219 (32.6)**a**	93 (13.9)**b**	216 (32.1)**a**	0.002	678 (20.0)**b**	1086 (32.6)**a**	297 (8.9)**c**	1266 (38.0)**a**	0.000
	Mean collected (±SD)	12.0 (8.7)	18.3 (11.8)	7.8 (6.9)	18.0 (10.2)		56.5 (30.3)	90.5 (57.9)	24.7 (17.1)	105.5 (48.8)	
	% total of *Culicoides*	3.8	4.0	2.7	2.8		18.9	15.5	12.4	14.7	
Sub. *Silvaticulicoides* [a]	Total collected	709 (23.3)	723 (23.8)	638 (21.0)	965 (31.8)	0.551	738 (16.0)	1380 (30.0)	505 (10.9)	1976 (42.9)	0.554
	Mean collected (±SD)	59.1 (76.1)	60.3 (75.1)	53.2 (70.5)	80.4 (92.9)		61.5 (33.3)	115.0 (92.0)	42.1 (30.2)	164.7 (70.6)	
	% total of *Culicoides*	18.8	13.1	18.6	12.5		20.6	19.8	21.7	22.8	
Sub. *Oecacta* [b]	Total collected	238 (17.1)**ab**	392 (28.2)**a**	199 (14.3)**b**	557 (40.1)**a**	0.017	339 (19.3)**ab**	473 (26.9)**a**	260 (14.8)**b**	683 (38.9)**a**	0.020
	Mean collected (±SD)	19.8 (11.3)	32.7 (18.9)	16.6 (15.4)	46.4 (45.6)		28.3 (32.2)	39.4 (32.9)	21.7 (30.2)	56.9 (53.9)	
	% total of *Culicoides*	6.3	7.1	5.8	7.2		9.5	6.8	10.9	7.9	
Males [c]	Total collected	124 (17.2)	214 (29.8)	107 (14.9)	272 (37.9)	0.056	95 (16.0)**b**	178 (30.0)**a**	50 (8.4)**b**	269 45.4)**a**	0.000
	Mean collected (±SD)	10.3 (16.9)	17.8 (24.6)	8.9 (12.2)	22.7 (34.6)		7.9 (9.1)	14.8 (9.3)	4.2 (3.6)	22.4 (18.1)	
	% total of *Culicoides*	3.3	3.9	3.1	3.5		2.7	2.5	2.1	3.1	

Numbers per row for the same centration doses followed by a different letters denote statistically differences at the 5% level based. Mix 2: Mixture 1:1 of geranyl acetone plus 6- methyl-5-hepten-2-one; Mix 3: Mixture 1:1:1 of octanoic plus nonanoic plus decanoic fatty acids.[a] Includes mainly *C. achrayi* and *C. picturatus* and in lesser extent *C. fascipennis, C. pallidicornis and C. fascipennis.*[b] Includes mainly *C. simulator* and in lesser extent *C. santonicus, C. poperinghensis, C. festivipennis, C. pictipennis, C. shaklawensis, C. vexans, C. tauricus, C. kibunensis, C. furcillatus and C. kurensis.* [c] Males pooled together. Other six *Culicoides* species discarded. P values >0.05 indicate no statistic difference.

Table 4. *Culicoides* midges collected with four traps impregnated with three different plant-derived treatments at 10% and 25% (w/w) during summer of 2013 at a sheep farm in the Basque Country region, Spain. 10 collections were made with each repellent.

		10% (w/w)						**25% (w/w)**					
Species	Treatment	DEET	Eucalypt.	Mix 1	Lavander	Control	P value	DEET	Eucalypt.	Mix 1	Lavander	Control	P value
No. of species collected		12	12	14	14	16		4	5	5	7	9	
Total *Culicoides* collected (%)		485 (13.2)**b**	613 (16.7)**b**	751 (20.5)**b**	693 (18.9)**b**	1121 (30.6)**a**	0.002	182 (9.9)**b**	219 (12.0)**b**	328 (17.9)**ab**	499 (27.3)**a**	600 (32.8)**a**	0.019
Mean collected (± SD)		48.5 (22.7)	61.3 (21.3)	75.1 (34.5)	69.3 (30.6)	112.1 (48.6)		18.2 (15.1)	21.9 (15.5)	32.8 (24.7)	49.9 (41.1)	60 (43.6)	
C. lupicaris	Total collected	148 (13.5)**b**	150 (13.6)**b**	233 (21.2)**b**	164 (14.9)**b**	403 (36.7)**a**	0.008	65 (9.5)**c**	71 (10.4)**c**	95 (13.9)**bc**	207 (30.3)**ab**	245 (35.9)**a**	0.002
	Mean collected (±SD)	14.9 (10.4)	15.0 (8.0)	23.3 (16.1)	16.5 (30.5)	40.3 (31.5)		6.5 (5.9)	7.1 (5.4)	9.5 (8.6)	20.7 (25.1)	24.5 (26.5)	
	% total of *Culicoides*	30.5	24.5	31.0	23.7	36.7		35.7	32.4	29.0	41.5	40.8	
C. obsoletus + *C. scoticus*	Total collected	93 (10.5)**c**	182 (20.4)**b**	170 (19.1)**bc**	174 (19.5)**bc**	270 (30.4)**a**	0.004	82 (11.8)**b**	92 (14.5)**ab**	145 (22.9)**a**	159 (25.1)**a**	214 (33.7)**a**	0.038
	Mean collected (±SD)	9.3 (5.3)	18.2 (6.0)	17.0 (8.8)	17.4 (7.1)	27.0 (13.9)		8.2 (12.1)	9.2 (6.4)	14.5 (13.6)	15.9 (9.9)	21.4 (14.9)	
	% total of *Culicoides*	19.2	29.7	22.6	25.1	24.1		45.1	42.0	44.2	31.8	35.7	
C. punctatus	Total collected	41 (11.0)	79 (21.6)	112 (30.5)	65 (17.5)	71 (12.3)	0.492	0	0	0	0	0	
	Mean collected (±SD)	4.1 (2.8)	7.9 (10.2)	11.2 (10.5)	6.5 (10.8)	7.1 (6.8)		0	0	0	0	0	
	% total of *Culicoides*	8.5	12.9	14.9	9.4	6.3		0	0	0	0	0	
Sub. *Silvaticulicoides*[a]	Total collected	109 (15.8)	103 (14.8)	150 (21.6)	153 (22.0)	179 (25.7)	0.456	0	0	0	0	0	
	Mean collected (±SD)	10.9 (8.3)	10.3 (6.8)	15.0 (11.9)	15.3 (12.4)	17.9 (6.9)		0	0	0	0	0	
	% total of *Culicoides*	22.5	16.8	20.0	22.1	16.0		0	0	0	0	0	
Sub. *Oecacta*[b]	Total collected	46 (19.6)**b**	31 (13.0)**b**	25 (10.7)**b**	39 (16.7)**b**	94 (40.1)**a**	0.026	35 (8.5)	47 (11.5)	80 (19.5)	119 (29.0)	129 (31.5)	0.127
	Mean collected (±SD)	3.8 (3.4)	2.5 (2.3)	2.1 (1.3)	3.3 (2.4)	7.9 (8.9)		3.5 (3.9)	4.7 (5.3)	8.0 (7.8)	11.9 (11.1)	12.9 (12.8)	
	% total of *Culicoides*	9.5	5.1	3.3	5.6	8.4		19.2	21.5	24.4	23.8	21.5	
Other[c] *Culicoides* spp.	Total collected	39 (13.7)	47 (16.4)	50 (17.5)	75 (26.0)	76 (26.4)	0.577	0	8 (25.8)	6 (19.3)	10 (32.3)	7 (22.6)	NA
	Mean collected (±SD)	4.0 (4.3)	4.7 (3.8)	5.1 (2.3)	7.5 (5.2)	7.6 (4.1)		0	0.8 (0.6)	0.6 (0.5)	1 (0.7)	0.7 (0.6)	
	% total of *Culicoides*	8.0	7.7	6.7	10.8	6.8		0	3.7	1.8	2.0	1.2	
Males[d]	Total collected	8 (8.8)	22 (23.4)	12 (12.2)	24 (25.3)	28 (30.1)	0.439	0	1 (8.0)	2 (17.2)	4 (33.1)	5 (42.4)	NA
	Mean collected (±SD)	0.7 (1.0)	1.8 (2.3)	0.9 (1.0)	1.9 (3.1)	2.3 (2.9)		0	0.1 (0.3)	0.2 (0.5)	0.4 (0.7)	0.5 (1.3)	
	% total of *Culicoides*	1.6	3.6	1.6	3.5	2.5		0	0.5	0.6	0.8	0.8	

Numbers per row for the same centration doses followed by a different letters denote statistically differences at the 5% level based. NA: Not analyzed; Mix 1: Mixture 1:1:1 of jasmine plus lavander plus rosemary. [a] Includes mainly *C. achrayi* and *C. picturatus* and in lessesrextent *C. pallidicornis*, *C. fascipennis* and *C. subfascipennis*.[b] Includes mainly *C. simulator* and in lesser extent *C. brunnicans*, *kibunensis*, *C. furcillatus*, *C. festivipennis*, *C. vexans*, *C. gejgelensis*, *C. kurensis* and *C. alazanicus*.[c] Includes other six *Culicoides* species. [d] Males pooled together. P values >0.05 indicate no statistic difference. NA: Not analized.

6. DISCUSSION

Of the 23 compounds evaluated, 13 were previously shown to be effective against various Culicidae mosquitoes (Amer and Mehhorn, 2006; Logan et al. 2010; Nerio et al. 2010; Campbell et al. 2011; Maia and Moore, 2011; Kazembe and Chaivba, 2012; Phasomkusolsil and Soonwera, 2012). Previous studies also showed that cypermethrin (Braverman and Chizov-Ginzburg, 1998; Calvete et al. 2010), DEET (Braverman et al., 1997; Braverman and Chizov-Ginzburg, 1998; Page et al. 2009), neem oil (Braverman and Chizov-Ginzburg, 1998, Braverman et al. 1999), lemon eucalyptus (Braverman et al., 1999), rosemary (Braverman and Chizov-Ginzburg, 1998) and fatty acids mixture (Venter et al. 2011) will repel *C. imicola*. Compounds that previously were shown to repel *C. sonorensis* were neem oil and lemon eucalyptus (Braverman et al. 2000; Trigg and Hill, 1996) and cypermethrin (Papadopolos et al. 2009, 2010). Picaridin (Carpenter et al.,2005), neem oil (Blackwell et al. 2004; Trigg, 1996) and 6-methyl-5-hepten-2-one (Bhasin et al. 2001) were shown to repel *C. impunctatus*. Martinez-de la Puente et al. (2009) showed that IR3535 will repel *Culicoides* species in general. Some compounds, e.g. 4-propyl-phenol, were shown to attract rather than repel *C. impunctatus* (Bhasin et al. 2001). While some studies showed that the oil of *Eucalyptus maculata* var. *citriodora* will repel *C. impunctatus*, from humans in the field (Trigg, 1996) and the *C. variipennis*, from humans in the laboratory (Trigg and Hill, 1996), Braverman et al. (1999) found that it can attract *C. imicola*.

The comprehensive evaluation of repellents should be based on laboratory as well as field data (Logan et al. 2010a). Although olfactometer and other laboratory based assays are effective in determining the responses of biting insects to odour stimuli under controlled conditions, field studies are considered more realistic and natural. Although olfactometry assays might evoke unnatural behaviour (e.g. insects may respond differently to the same odour stimuli when walking as opposed to flying), and elicit chemotactic and chemokinetic responses (Kennedy, 1977). Logan et al. (2010a) demonstrated the results between laboratory and field trials to be comparable. Comparison of the results obtained with these two laboratory assays showed some discrepancies. While the olfactometer assays indicate DEET and mixture 3 to be superior to the essential oils lemon eucalyptus, lavender and mixtures 2-4, the landing-assays were, however, rather disappointing (Table 2, Fig. 1). This apparent discrepancy could be attributable to the fact that in the olfactometer the midges were in a confined space whereas in the landing-assays they had

capacity of flight. In the current study, the compounds evaluated in the field were selected based on two different laboratory assays and include products which were previously shown to be successful in repelling mosquitoes.

In both laboratory assays used repellence were determined for relatively short exposure periods (2 seconds to minutes) and released of the potential repellent. During these first seconds and/or minutes after application high volatility, achieving a strong efficacy for a relative short time, occurred. This initial strong repellent effect seems to become less effective over time as was shown with light traps collections. A similar observation was made with mixture 2 (chemical formulation) in the field. Although it repels more than 85% of the midges in the olfactometer and landing bioassays it appeared to be rather infective in the light trap study. Similar results were obtained by Logan et al. (2010b) with mosquitoes in arm cage experiments that showed this mixture to be relatively effective over short time periods but that improvements to achieve slow release over long periods of time will be required. It must also be kept in mind that while the laboratory assays was conducted with *C. obsoletus*, the light trap studies involved a range of different species.

With nonanal, mixture 3 and cypermethrin less than 70% of the midges moved towards any of the two arms within the allocated 4 min (Table 2). This could be attributed to a potential toxic effect of these compounds, as was recorded for *C. nubeculosus* and the insecticide cypermethrin (Papadopoulus et al. 2010).

Despite some limitations, light traps will be appropriate for screening putative repellents before testing them on animals where these products may cause adverse allergic reactions such as skin irritation. The use of livestock may be expensive, in some cases inhumane and entails some difficulties in terms of animal management. Despite the fact that light traps are an artificial collection method that does not always reflect the true biting rate on animals (Viennet et al. 2011), the mixture of fatty acids was as effective as DEET in repelling *Culicoides* species from the traps. It was also shown that lemon eucalyptus extracts may be as effective as DEET in repelling *Culicoides* midges.

Although the majority of species or groups studied here responded similarly to repellents tested, some grouping e.g. pooled males, *C. punctatus* and particularly the subgenus *Silvaticulicoides*, comprising of *C. picturatus* (65% of the total captures), *C.*

achrayi (32%) and in lesser extent *C. fascipennis, C. pallidicornis* and *C. subfasciipennis* (3%), seem to be more resilient to some of the repellents. Although it was shown that these species will feed on mammals (Ninio et al. 2010; Lassen et al. 2012; Santiago-Alarcon et al. 2012; Pettersson et al. 2013) their role as disease vectors are still unknown. More relevant are the promising results obtained with *C. obsoletus/C. scoticus* and *C. lupicaris/C. pulicaris,* both these groups are of immense importance as potential vectors of arboviral diseases (Mellor et al. 2000; Carpenter et al. 2008). Recent studies show that *C. punctatus,* an abundant and ubiquitous midge in Europe, can play a greater role in the epidemiology of SBV and BTV in Europe than was previously suspected (Larska et al. 2013; Meiswinkel et al. 2013).

DEET is considered the most common and effective insect repellent used over the last 60 years (Fradin, 1998). It was shown that the efficiency of DEET at 15% doses against *C. imicola* can last from a few hours (Braverman et al. 1997, 2000) to as long as eight hours (Page et al. 2009). Although not of statistical significance, traps baited with the mixture of fatty acids collected fewer midges than the traps baited with DEET (Table 3 and 4).

Results obtained with plant-based repellents suggested that the oil of lemon eucalyptus will perform better at high doses (25%), reducing the collections 2,7 times compared to the controls. Although plant-derived repellents at 10% were statistically different from and collected 1.5 to 1.8 times fewer midges than the control trap, DEET collected 2.3 times fewer midges than the control. The other plant extracts in the present study although of statistical significance seemed to be less effective. The active ingredient of *Eucalyptus maculata* var. *citriodora* (PMD: p-menthane-3,8 diol) tends to be less volatile than other natural plant derivatives and can provide good protection for several hours (Fradin and Dalay, 2002; Maia and Moore, 2011).

7. CONCLUSIONS

To our knowledge, this is the first evaluation of different repellents against livestock-associated *Culicoides* vector species in Iberian Peninsula. Of the 23 compounds at least three showed promising results with the methodologies used. In chemicals, the widely used insect repellent DEET (*N,N*-diethyl-3-methylbenzamide) and the mixture of fatty acids (octanoic, nonanoic and decanoic organic acids) were shown to be effective. In plant-derived repellents,

the activity of lemon eucalyptus oil (*Eucalyptus maculata* var. *citriodora*) in both cases high doses perform better in the field. The discrepancies between laboratory and field results in this study highlight some of the problems and factors that can influence the results in evaluations. Despite some discrepancies laboratory based assays could serve as a preliminary approach to screen repellents to be evaluated in the field.

Taking into account the apparent repellent effects of the mixture of fatty acids it is recommended that this product be further evaluated. The skin reactions in animals, in vivo testing, synergistic formulations, new doses and release rate are some of the aspects that need to be determined. Novel designs of formulation technologies with synergistic effects that may result in higher bioactivity, e.g. mixing essential oils with synthetic blends as well as new formulations and dispositives are needed.

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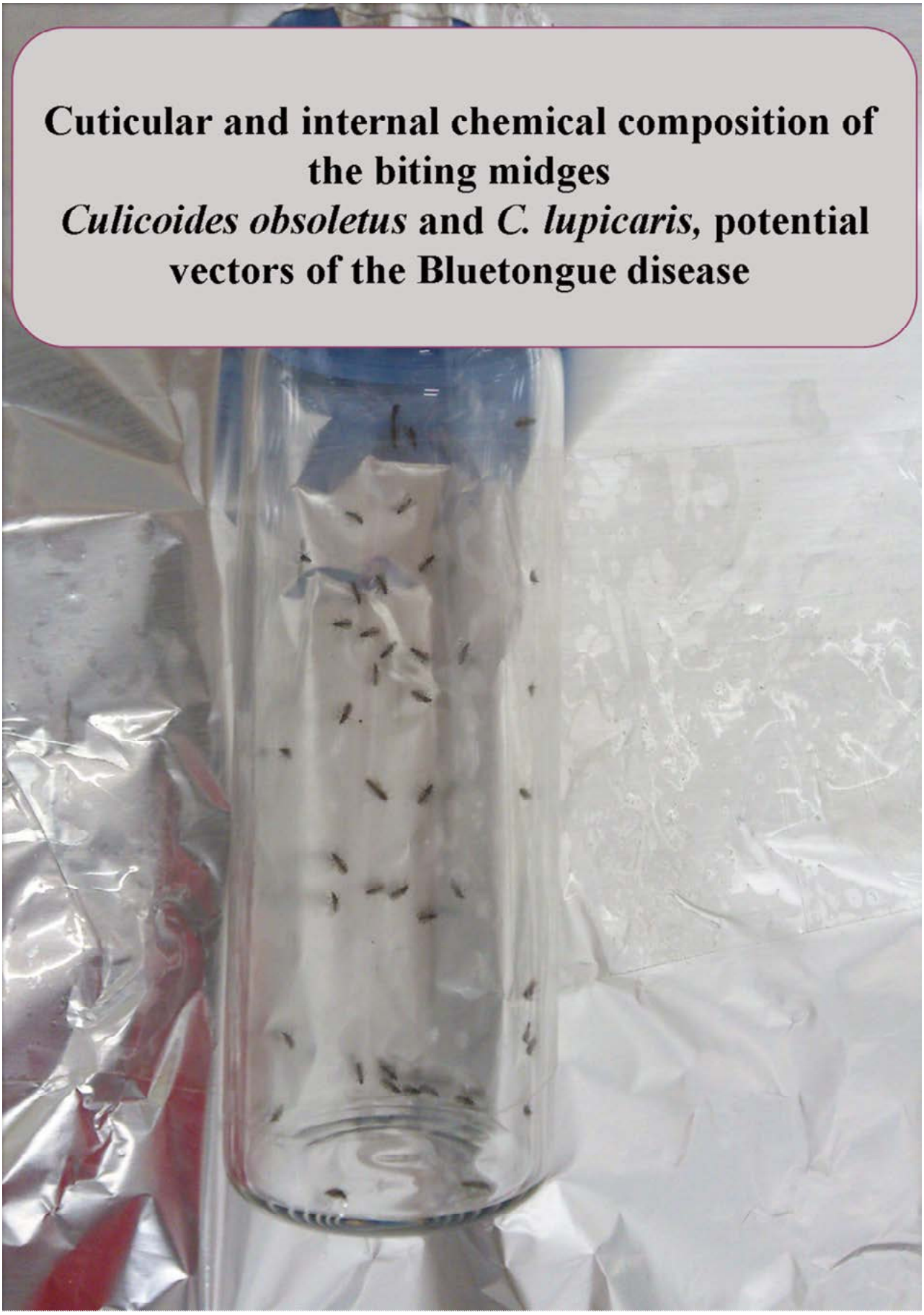
CHAPTER 5

Version of the chapter 5 based on the following publication:

Cuticular and internal chemical composition of the biting midges *Culicoides obsoletus* and *C. lupicaris* (Diptera: Ceratopogonidae), potential vectors of the Bluetongue disease (Submitted to Insect Science)

Mikel González, Sergio López, Gloria Rosell, Ángel Guerrero and Arturo Goldarazena

Cuticular and internal chemical composition of the biting midges *Culicoides obsoletus* and *C. lupicaris*, potential vectors of the Bluetongue disease



CUTICULAR AND INTERNAL CHEMICAL COMPOSITION OF THE BITING MIDGES *CULICOIDES OBSOLETUS* AND *C. LUPICARIS*, POTENTIAL VECTORS OF THE BLUETONGUE DISEASE

1. ABSTRACT

The chemical profile of the cuticle and internal tissues of four female species of *Culicoides* by coupled gas chromatography-mass spectrometry is reported. The chemical composition of *Culicoides obsoletus* and *C. lupicaris*, vectors of diverse viral diseases, has been compared with that of other biting midges, such as *C. kibunensis* and *C. fascipennis*, and the non-biting midge *Forcipomyia bipunctata*. A total of 67 compounds belonging to seven major chemical classes were identified in cuticular and internal tissues in hexane extracts. The most dominant compounds include fatty acids (C6-C20), with C16:0, C16:1 and C18:1 as dominant, branched hydrocarbons (C29 to C38 mono/di/trimethylalkanes), linear hydrocarbons (C15 to C33, odd chain carbons as predominants), terpenes (squalene, terpenic alcohol), steroids (cholesterol), aldehydes (saturated even chain from C9 to C10 and C20 to C30), esters (derived from primary and secondary alcohols), and as minor constituents alcohols (saturated C16 of even chain), lactones (isocoumarin), diterpenes esters (geranyl geraniol acetate) and a single alkene. The chemical composition varies depending on the species and on the cuticular extracts (internal or external). The relative contents of linear and branched hydrocarbons, and aldehydes was high in cuticular extracts but practically absent in internal tissues. In contrast, fatty acids, terpenes and steroids were, in general, high in internal tissues. Qualitative analysis of four gonotrophic status of *C. obsoletus* and *C. lupicaris* to try to correlate the estimated age of the wild midges with the amounts of the major compounds of their cuticular and internal extracts showed no significant differences except for squalene and cholesterol. The results are discussed and compared to other *Culicoides* midges and/or other mosquito related species.

2. KEY WORDS *Culicoides obsoletus, Culicoides lupicaris,* Biting midges, Chemical characterization, Cuticular composition, Internal tissue composition

3. INTRODUCTION

Biting midges of the genus *Culicoides* are tiny haematophagous insects worldwide known because adult females have a demonstrated role as vectors of diverse viral diseases of veterinary and economical interest, such as the Bluetongue virus (BTV) (Mellor et al. 2000), the African-horse sickness virus (AHSV) (Coetzer and Guthrie, 2004), the Epizootic haemorrhagic disease virus (EHDV) (Ruder et al. 2012) or the Schmallenberg virus (SBV) (Rasmussen et al. 2012; Larska et al. 2013). Knowledge of the taxonomy, epidemiology and bioecology of these insects has received a great interest in the last years after several BTV outbreaks in the Mediterranean regions and other countries of North and Central Europe (Wilson and Mellor, 2009). The sanitary prophylaxis of these diseases has been mainly based on the use of repellent and insecticidal formulations followed by extensive vaccination upon intense surveillance programs (MARM, 2008).

Insects cuticle plays an important role in numerous biochemical, physiological and semiochemical processes (Blomquist et al. 1987; Steiger et al. 2007; Gołębiowski et al. 2011; Pedrini et al. 2013), particularly in inter and intraspecific communication (Wyatt, 2003; Böröczky et al. 2009). Cuticular compounds may also act as inducer of insect resistance to entomopathogens (bacteria, fungi) (Smith and Grula, 1982; Sosa-Gomez et al. 1997; Howard and Lord, 2003), as inhibitors of fungal attachment (Pedrini et al. 2013) and as antimicrobial agents (Gołębiowski, 2012a; Urbanek et al. 2012). In addition, cuticular hydrocarbons were proposed as a taxanomic tool for insect identification (Jackson and Blomquist, 1976; Lockey, 1976) and to differentiate phylogenetically closely related species of medical interest (Mahamat and Hassanali, 1998; Phillips et al. 1988; Horne and Priestman, 2002; Caputo et al. 2005). As inter and intraspecific communication agent in insects, cuticular hydrocarbons have been used as species, mate, kin, nest-mate or castle recognition cues and reservoir for pheromones, chemical defense and thermoregulation (Blomquist et al. 1987; Steiger et al. 2007; Pedrini et al. 2013). However, the role of cuticular compounds as intraspecific recognition cues in mating behaviour of *Culicoides* spp. remains unknown. In only three species, *Culicoides impunctatus*, *C. melleus* and *C. nubeculosus* there is some evidence of the presence of pheromone compounds. Thus, females of *C. impunctatus* appear to produce an aggregation or recruitment pheromone to attract individuals of the same sex (Blackwell et al. 1994). In *C. melleus*, Linley and Carlson (1978) reported that a mixture of methyl substituted *n*-alkanes of C22 and C23 found on the female cuticle could be a contact pheromone stimulating males to attempt

copulation. The same authors later reported the first cuticular hydrocarbons in *C. melleus* and *C. variipennis* and their biological importance as putative sex pheromones (Linley and Carlson, 1982). *Culicoides nubeculosus* females, in turn, produce *n*-heptadecane as sex pheromone to attract male congeners for mating (Mordue et al. 2002). Other cuticular compounds of five species of *Culicoides* from non-Palearctic regions identified as mixtures of fatty acids (FAs) have been reported but their biological significance has not been determined (Jin-Hua et al. 1994). We present herein a complete chemical profile of the biting midges *C. obsoletus* and *C. lupicaris,* the two most abundant species in Northern Spain and major vectors of BTV in North-Central Europe, in comparison to *C. kibunensis* and *C. fascipennis,* other common species of unknown medical interest, and the non-biting midge *F. bipunctata.* The results are discussed in terms of their possible implication in the chemical communication between *Culicoides* species and in comparison to Culicidae insects, whose females are also blood-feeders of vertebrates.

4. MATERIAL AND METHODS

4.1. Insect collections

Ceratopogonid specimens were collected between May 2011 and September 2012 in a sheep farm located in Elguea (N 42º 55´ 59´´, E 02º 30´ 51´´) in the province of Alava, Basque Country, Northern Spain. Dynamic populations of several species of *Culicoides* biting midges had been previously established in this farm (González et al. 2013). Midges were wild-caught using an ultraviolet CDC light trap model 1212 (J.W. Hock, Gainesville, USA,) containing a cubic mesh nest (30x30x30 cm) to minimize insects damage and adapted to keep them alive.

4.2. Insects classification

Insects were maintained in the laboratory in larger cubic mosquito cages (90x90x90 cm) without any feeding source. Midges were anesthetized under a carbon dioxide stream, manually aspirated and classified to species level under a stereo-microscope (10-40x). Females of five species of midges, i.e. *Culicoides obsoletus, C. lupicaris, C. fascipennis, C. kibunensis* and the non-biting midge *Forcipomyia bipunctata,* were identified according to their wing pattern (González and Goldarazena, 2011). *Culicoides obsoletus* and *C. lupicaris* were grouped by their abdomen characteristics as: 1)

non-pigmented (abdomen with a homogeneus palish pigmentation or colourless), 2) blood-engorged (abdomen with blood), 3) gravid (abdomen with mass of eggs), and 4) pigmented (abdominal tergites with purple pigmentation) (Dyce, 1969) (Fig. 1).

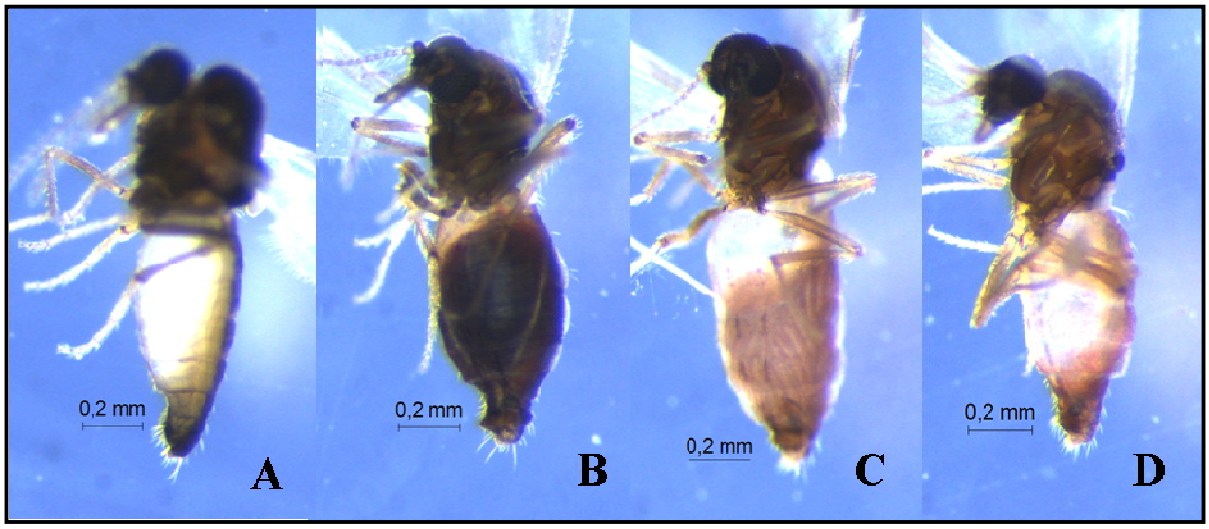


Fig. 1. *Culicoides obsoletus* physiological stages **(A)** Non-pigmented **(B)** Blood-engorged **(C)** Gravid and **(D)** Pigmented.

4.3. Preparation of extracts

4.3.1. Adults of unknown age

Specimens of different ages were pooled and grouped in three different samples (3 replicates) each one containing 40-60 individuals. Cuticular extracts were obtained by immersing the insects in glass vials containing 1,5 ml of analytically pure hexane (SupraSolv, Merck, Darmstadt, Germany) sufficient to cover the entire body of the insects. After 1 h, the extracts were stirred gently by hand twice during ca. 3 s at room temperature (25 ± 3ºC), and the extracts were pipeted to conical vials with caution to avoid damage of female carcasses. These individuals were transferred to a glass-mortar (Afora, Fisher Scientific, Madrid, Spain), where their tissues were ground during 15 s in hexane to extract the internal content. After five minutes, the supernatant was transferred into another new vial. Both extracts (named hereafter as cuticular and internal extracts) were stored at -20ºC until analysis. Prior to GC/MS analysis, extracts were evaporated to dryness with a gentle flow of nitrogen, diluted with 10 µl of hexane and 2 µl of the new solution were injected in the GC/MS system. Data were analyzed according to their peak areas relative to the total area of all peaks and the means of the three replicates were categorized by symbols according to their abundance: t (trace): ≤0.1%; +: 0.1-1%; ++: 1-5%; +++: 5-10%; ++++: 10-20%; +++++; ≥ 20%. Only peaks that appear in two of the three samples were included

in the analysis and compounds present only in trace amounts in all species and tissues were excluded from analysis.

4.3.2. Adults grouped by their physiological status

A second stock of *C. obsoletus* and *C. lupicaris*, sorted by their abdomen characteristics as cited above (see Insect classification) was also considered for analysis. Groups of five or ten individuals of each species and physiological status were extracted for 45 min at room temperature (22ºC ± 3ºC) (N=3-6 replicates depending on the availability of each stage) following the same protocol shown above. The extracts were concentrated to dryness and diluted with 10 µl of a 4 ng/µl solution of the IS (Internal Standard: pure *n*-pentadecane) in hexane for the 5-insect extracts, and the same volume of a 8 ng/µl solution of the IS for the 10-insect extracts. Two µl of these solutions were injected in GC-MS and quantification of the compounds was based on their peak areas relative to that of the IS.

4.4. Equipment

Samples were injected in splitless mode into a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain) in the electron impact (EI) mode at 70 eV. Helium was the carrier gas and the column was an HP-5MS (30 m x 0.25 mm x 0.25 µm) (Agilent Technologies, Madrid, Spain). The oven temperature program was as follows: injection at 60°C (1 min), and program of 5ºC/min to 180ºC, 2ºC/min to 200ºC, 5ºC/min to 270ºC (hold 20 min), and 5ºC/min to 300ºC (hold 10 min). A sample of each extract was also analyzed on a SPB-20 capillary column (30 m x 0.25 mm x 0.25 µm) (Supelco, Bellefonte, PA, USA) under similar GC conditions as above. For chemical ionization (CI) analysis, samples were injected in an Agilent 5973 Network MSD coupled to an Agilent GC 6890 Series using the HP-5MS column and methane as the reagent gas under the same chromatographic conditions as for the EI mass spectra.

4.5. Identification of compounds and statistical analysis

Compounds were identified by comparison of their MS and retention indexes with those of authentic standards and/or by those already described in the literature (Adams, 2007; NIST Registry of Mass Spectral Data, 2013). Determination of the branching position of methyl substituted alkanes was based on the fragmentation patterns reported (Nelson, 1972) and supported by their CI-MS. PRIMER v6 software package was used to determine the Bray-Curtis similarity index, a statistic parameter used to measure the compositional similarity among different elements based on the presence or absence of the major chemicals identified (compounds in trace amounts were excluded from the analysis) (Bray and Curtis, 1957). For significant differences of the mean amounts of compounds in midges at different gonotrophic stages, non-parametric Kruskal-Wallis tests followed by Bonferroni corrections were conducted (P=0.05).

5. RESULTS

5.1. Composition of cuticular and internal extracts

A total of 67 differentiated compounds in 63 peaks have been identified (55 in cuticular washes and 43 in internal tissues), some of them tentatively (Table 1). The compounds include 27 hydrocarbons (13 *n*-alkanes, 4 monomethylalkanes, 7 dimethylalkanes, 2 trimethylalkanes, and one alkene), 14 FAs (8 saturated and 6 unsaturated), 9 esters (7 saturated, 2 unsaturated), 8 aldehydes (saturated), 4 terpenes, one steroid, one alcohol and one lactone. The relative amount of the different chemical classes is shown in Figs. 2-3. The contents of aldehydes and linear and branched hydrocarbons are higher in the cuticle than in internal tissues whereas terpenes, steroids and FAs resulted, in general, more abundant in internal extracts.

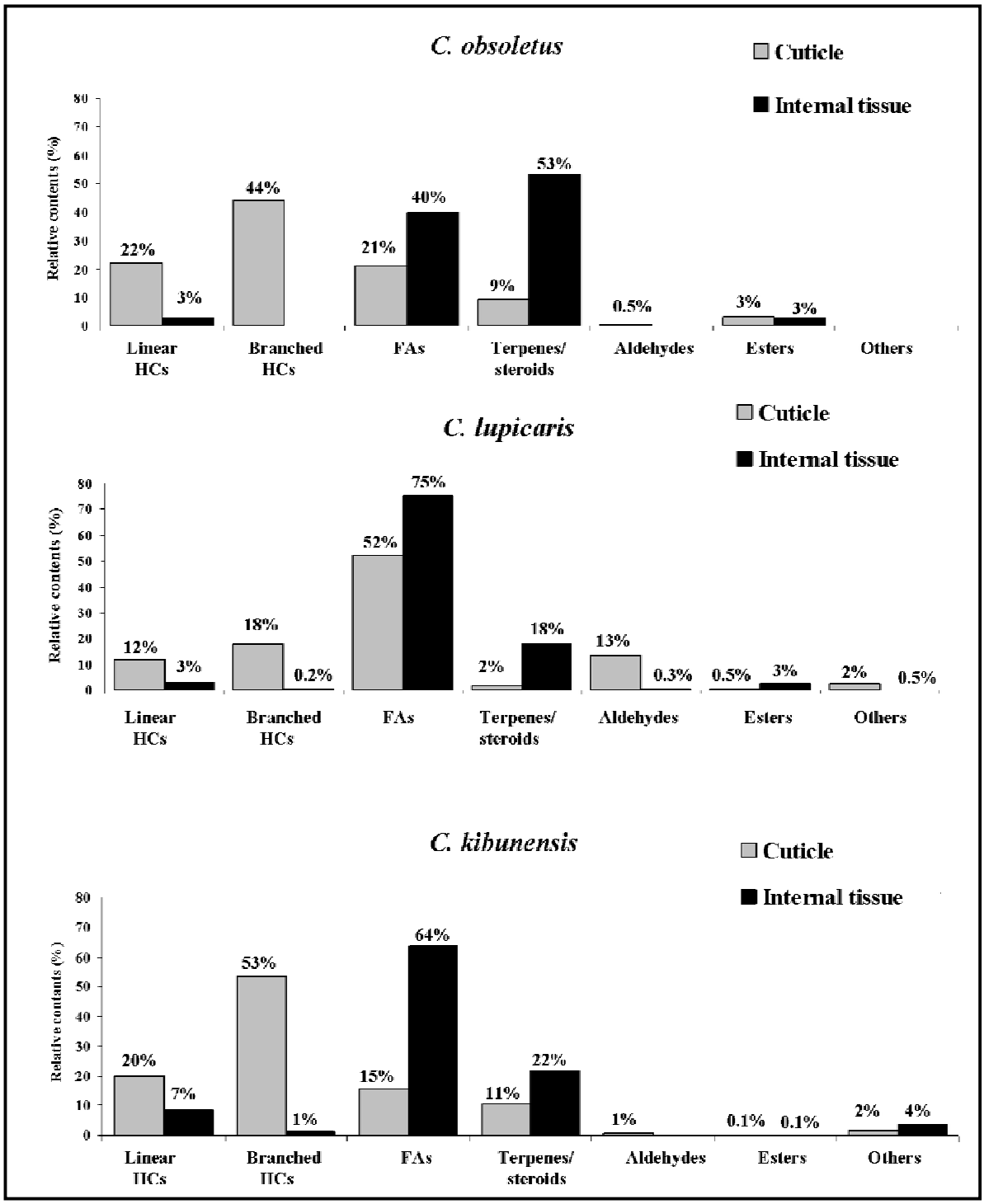


Fig 2. Relative contents of the main chemical classes of compounds extracted from the biting midges *C. obsoletus*, *C. lupicaris* and *C. kibunensis*. In gray, composition of cuticular washes; in black composition of internal tissue extracts.

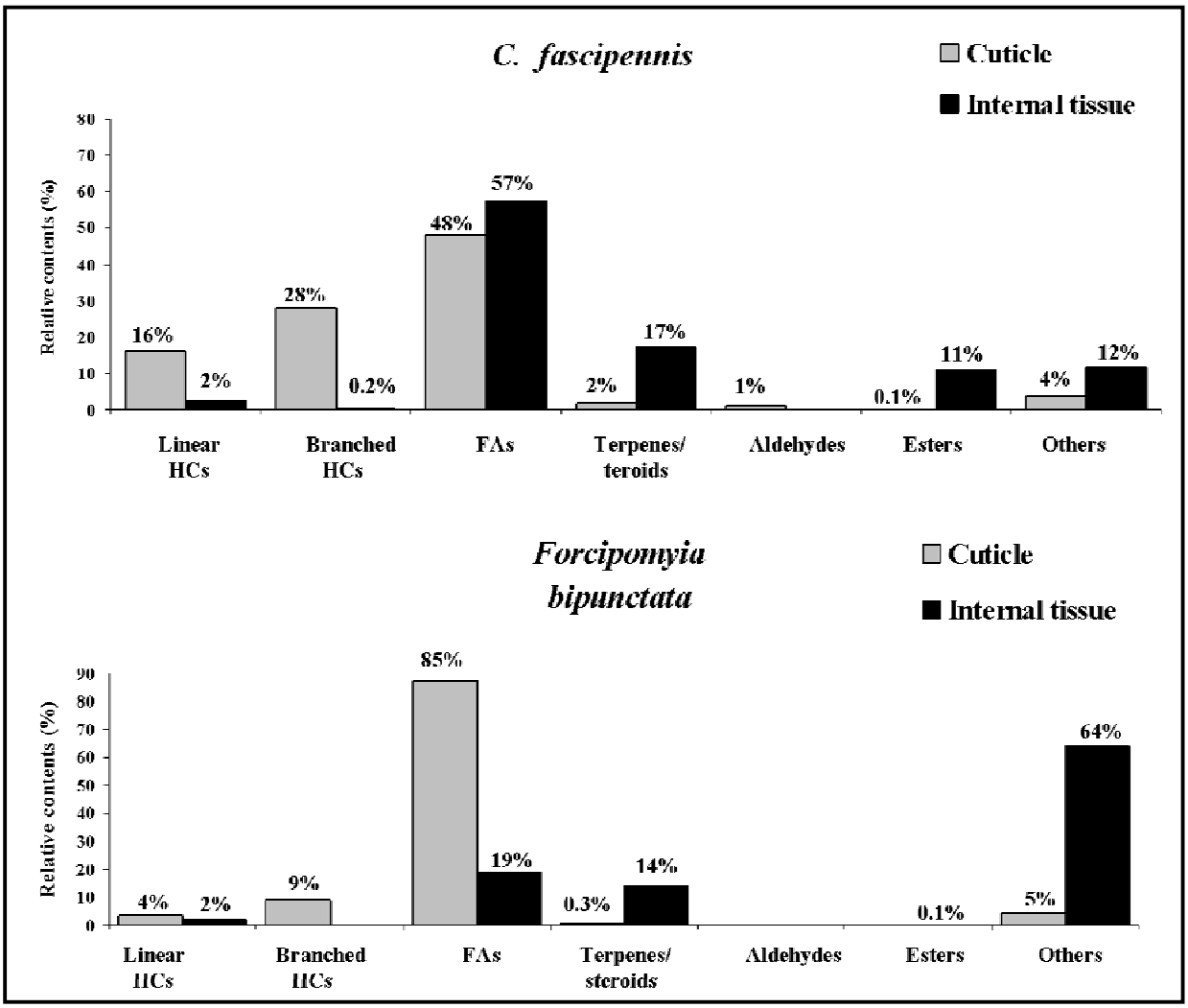


Fig. 3. Relative contents of the main chemical classes extracted from midges *C. fascipennis* and *F. bipunctata*. In gray, composition of cuticular washes; in black composition of internal tissue extracts.

Hydrocarbons. The main chemical constituents of the midges in either tissue were odd straight-chain hydrocarbons from C17 to C29 (Table 1). The pattern for distribution of linear alkanes in the cuticle follows the trend: *C. obsoletus* (C27>C17>C25>C23>C19>C29>C21), *C.lupicaris* (C17>C25>C19>C27>C23>C29>C21>C28) (Figs. 4-5). C27 (0.46 ± 0.12 ng per specimen) was the most abundant linear alkane in the former midge and C17 (0.47 ± 0.08 ng) in the latter with *n*-henicosane (C21) being the less common odd straight-chain hydrocarbon. Even straight-chain hydrocarbons (C12-C28) were very minor constituents, accounting as trace amounts or absent in many samples of all studied species. Monomethyl-, dimethyl-, and trimethyl-substituted hydrocarbons in different positions were detected in long chain hydrocarbons from C32 to C38 with 11,14 + 13,16-diMeC35 and 13-/15-/17-MeC35 being predominant. Interestingly, the former chemicals were particularly present only in the cuticle of all midges, not in the internal tissues. Alkenes were not detected in *Culicoides* biting

midges but *Forcipomyia* cuticular washes showed the presence of *n*-heptadecene in detectable quantities.

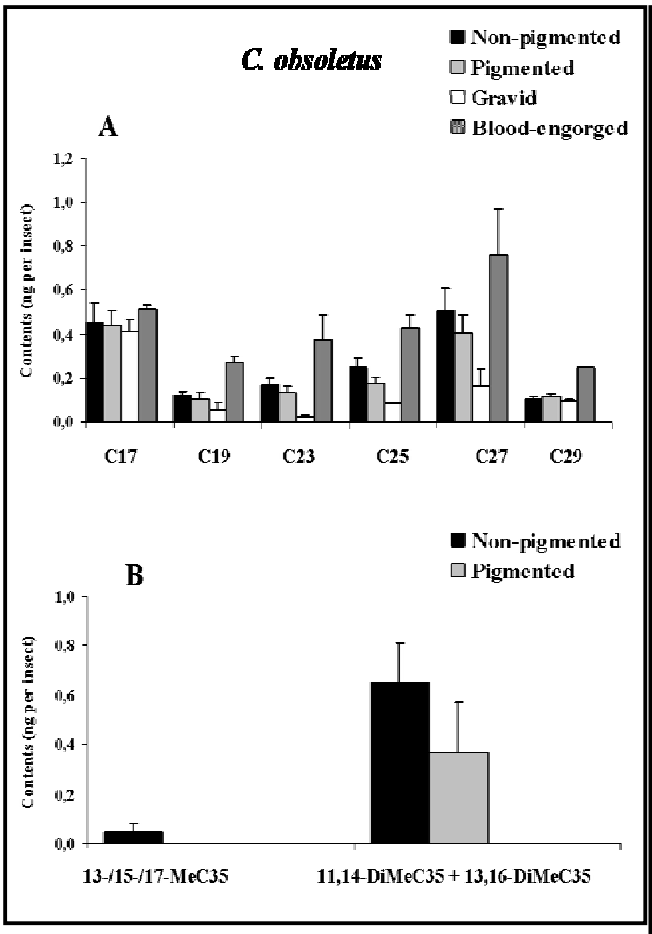


Fig. 4. Cuticular extracts of *C. obsoletus* **(A)** linear hydrocarbons **(B)** branched hydrocarbons. Only major compounds (>1%) with odd number of carbons are included. Vertical lines represent ±SE. Sample size: Non-pigmented (N=3), Pigmented (N=3), Gravid (N=3), Blood-engorged (N=3).

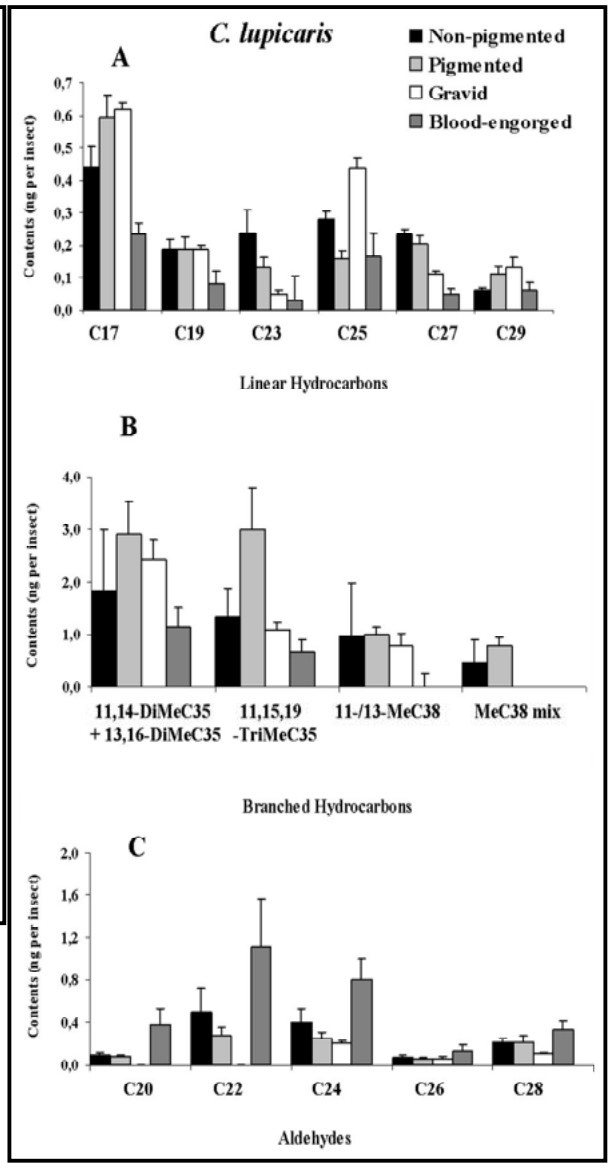


Fig. 5. Cuticular extracts of *C. lupicaris* **(A)** linear hydrocarbons **(B)** branched hydrocarbons **(C)** aldehydes. Only major compounds with odd number of carbons are included. Vertical lines represent ±SE. Sample size: Non-pigmented (N=3), Pigmented (N=6), Gravid (N=3), blood-engorged (N=4).

Fatty acids. As terpenes, FAs were in general more abundant in internal extracts. A total of 14 FAs ranging from C6 to C20 have been detected in the present study: 8 saturated FAs corresponding to C6, C8, C9, C10, C14, C16, and C18; three monounsaturated assigned as C16:1, 17:1 and 18:1 (oleic acid), one diunsaturated as C18:2 (linoleic acid), and a polyunsaturated compound identified as a mixture of arachidonic and dihydroarachidonic acids (Table 1). In all *Culicoides* internal tissues and in higher amounts than in cuticular washes, four FAs dominated: hexadecanoic acid (C16:0), hexadecenoic acid (C16:1), and oleic (C18:1) and linoleic acid (C18:2). In the non-biting midge *F. bipunctata,* in turn, the latter two compounds were predominant in both types of extracts, particularly in the cuticle. In addition, as cited, shorter saturated FAs were also detected as minor components, higher in internal tissues than in the external cuticle.

Contents of the FAs vary enormously within the gonotrophic stages considered. Thus, in internal tissues of blood engorged *C. lupicaris* the mean contents of C16:0 was 0.90 ± 0.69 ng per insect, C16:1 was present in 2.67 ± 1.87 ng, and C18:1 + C18:2 in 2.55 ± 2.11 ng, whereas in pigmented and gravid females the corresponding amounts were C16:0 2.09 ± 0.33 and 0.80 ± 0.20; C16:1 3.93 ± 1.64 and 0.50 ± 0.10; and C18:1 + C18:2 8.60 ± 2.68 and 2.10 ± 0.80, respectively. Non-pigmented specimens showed much lower amount of FAs with only 0.15 ± 0.05 ng per insect of C16:1.

Esters. Two isopropyl esters of medium-chain FAs (C14 and C16) prevailed, particularly in the internal tissues of the biting midges whereas the non-biting midge *F. bipunctata* lacked any ester in either tissue (except a minor amount of methyl 9-hexadecenoate). Ethyl hexadecanoate, ethyl octadecenoate and two long-chain acetates of C18 and C20 were also occasionally present in some extracts.

Aldehydes. Short-chain aldehydes were scarcely present in the extracts with nonanal and decanal being the only ones detected in minor amounts in one or both extracts depending on the *Culicoides* species. Long-chain (>C20) even-numbered aldehydes were much more abundant in the cuticle of *C. lupicaris,* particularly docosanal, tetracosanal and octacosanal, although they were practically undetected in the other midges. Surprisingly, in *C. obsoletus* and *C. kibunensis* only traces (≤0.1%) of the aldehydes were detected and none in *F. bipunctata.*

Terpenes, steroids and other compounds. Squalene, the precursor of cholesterol, was present in variable amounts in almost all extracts of *Culicoides* spp. but not in *Forcipomyia*. Cholesterol was the most abundant compound in almost all extracts with, again, the exception of *F. bipunctata* in which it was detected as a minor component in the cuticular extract. Both compounds were, in general, more dominant in internal tissues than in the cuticle (Fig. 7). Geranyl-geraniol acetate and a terpenic alcohol were detected in the extracts of some *Culicoides* spp. but mainly in the internal tissue of *F. bipunctata.* Alcohols were scarcely detected in *Culicoides* biting midges, with only small amounts of 1-hexadecanol being noticed in *C. obsoletus* and *C. lupicaris*. Among lactones, only isocoumarin was detected in significant amounts in both types of extracts of *F. bipunctata* (Table 1).

5.2. Composition of cuticular and internal extracts at different gonotrophic stages

Abundance of the most representative compounds of the cuticular profile of *C. obsoletus* (hydrocarbons) and *C. lupicaris* (hydrocarbons and aldehydes) at different gonotrophic stages are given in Figs. 4,5. The cuticular and internal tissue contents of cholesterol and squalene of both species are also presented in Figs. 6,7. In most cases, the mean amounts ± SE of the identified compounds in any of the gonotrophic stages studied were not statistically different. Only the amounts of cholesterol in the internal tissue extracts of gravid and blood-engorged, and squalene in gravid *C. obsoletus* females were significantly higher than in the other gonotrophic stages (Kruskal-Wallis test, $P<0.05$) (Fig. 6). In contrast, in *C. lupicaris* the contents of cholesterol and squalene in the cuticular extracts of gravid and blood-engorged females, respectively, were the only values significantly higher than the corresponding to the other gonotrophic stages (Fig. 7). It should be noted the lack of correlation between data of Figures 4-7 and Table 1. In the figures the mean contents per insect at specific gonodotrophic stages of some key compounds previously identified are presented. Table 1, in turn, shows a qualitative analysis of the chemical composition of the extracts relative to the total area of all peaks.

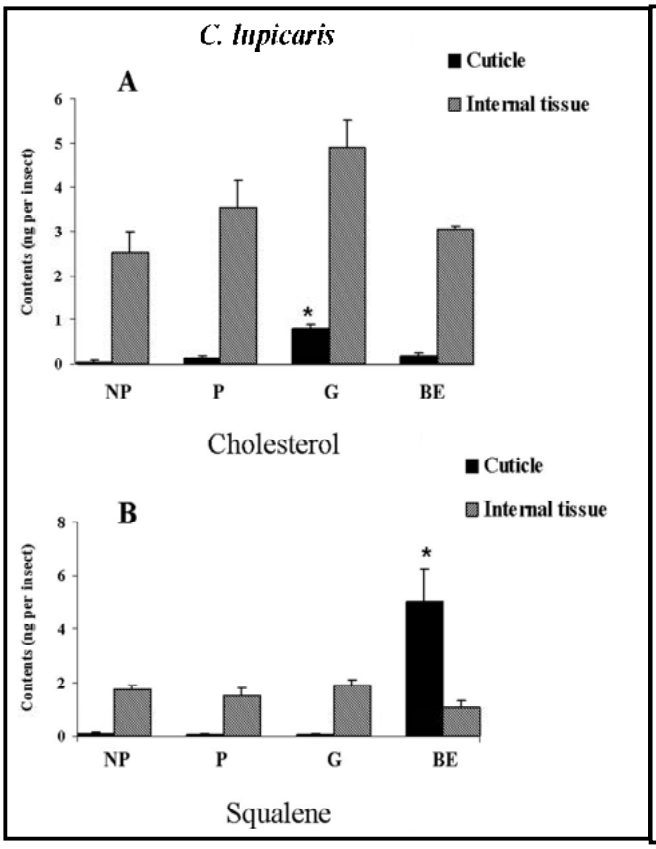


Fig. 6. Cuticular and internal extracts of *C. obsoletus* **(A)** cholesterol **(B)** squalene.Vertical lines represent ± SE. The The asterisk represents significant differences among Groups (Kruskal-Wallis test, P<0.05). Sample size: Non-Pigmented (N=3), P: Pigmented (N=3), G: Gravid (N=3), BE: Blood-engorged (n=3).

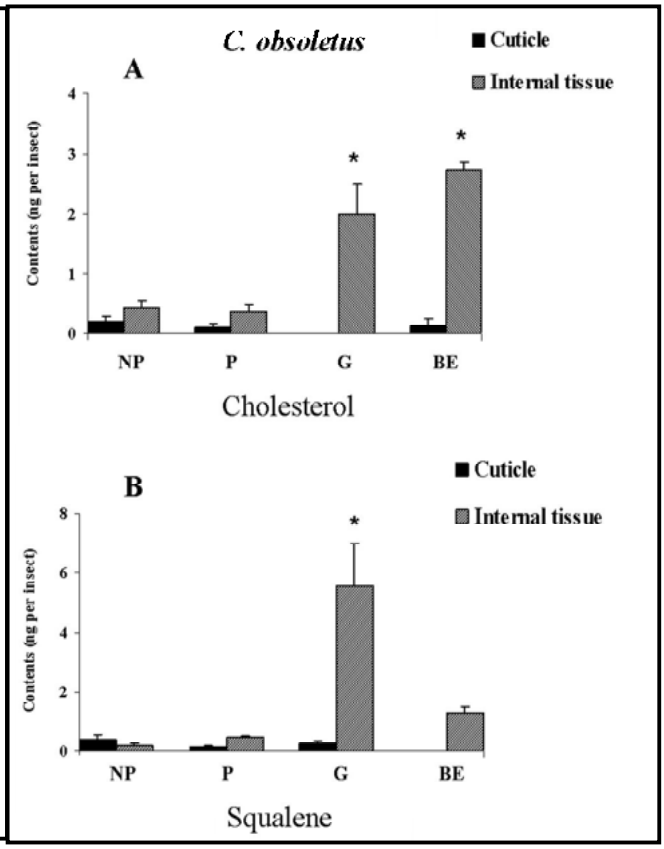


Fig. 7. Cuticular and internal extracts of *C. lupicaris* **(A)** cholesterol **(B)** squalene. Vertical lines represent ± SE. The asterisk represents significant differences among groups (Kruskal-Wallis test, P<0.05). Sample size: NP: Non-pigmented (N=3), P: Pigmented (N=6), G: Gravid (N=3), BE: Blood-engorged (N=4).

5.3. Morphological/chemotaxonomy similarity

Based on the compounds identified in both types of extracts, the clustering dendrograms showed in Figure 8 were built. The dendrogram of cuticular washes (Fig. 8A) shows two main groups. The first group is represented by *C. fascipennis, C. obsoletus*, followed by *C. kibunensis* and *F. bipunctata* being separated from the other species. The dendrogram profile of the internal compounds (Fig. 8B) clearly shows two differentiated groups, each one corresponding to midges of the two genus considered (*Culicoides* or *Forcipomyia*).

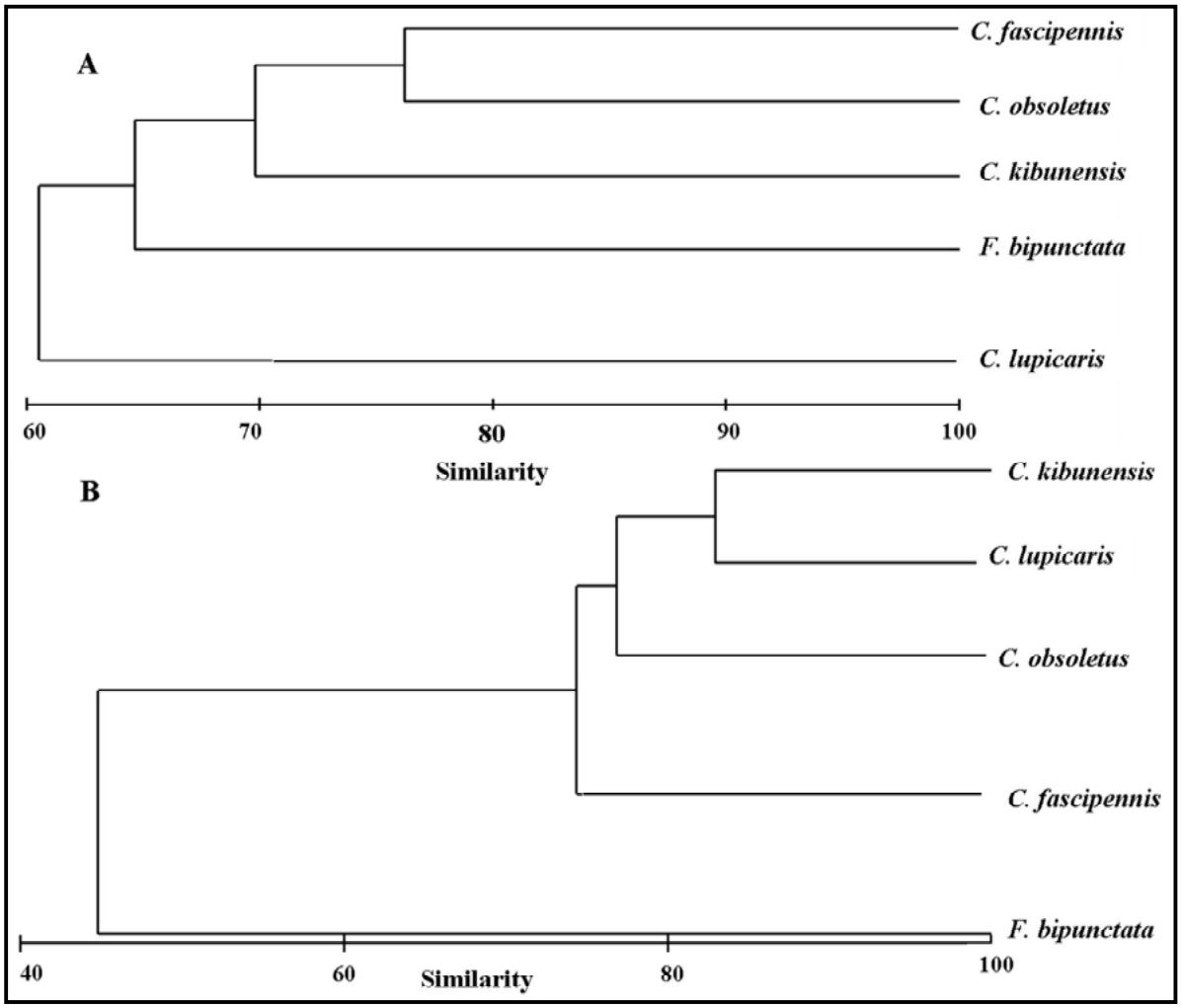


Fig. 8. Dendrogram based on Bray-Curtis similarity group average between chemical compounds profile of the five species studied **(A)** From cuticular extracts **(B)** From internal tissue extracts. Compounds in trace amounts were excluded from the analysis. Chemical profiles of species of the same genus are more similar (closer) on internal extracts but in cuticle extracts *C. lupicaris* is quite separated from the remaining species. The shorter the distance between profiles, the greater the similarity.

6. DISCUSSION

This study shows that the cuticular profile of *Culicoides* spp., two of them potential vectors of BTV, is a complex mixture of diverse families of chemicals being predominant linear and methyl-branched alkanes, FAs, terpenes, steroids, aldehydes and esters. Quantitatively, there is a wide variability between the cuticular and internal tissue extracts within the same species. The amount of many identified compounds may be affected by environmental factors, such as habitat, temperature, relative humidity, season and developmental stage (Toolson and Hadley, 1979; Toolson, 1982; Gołębiowski et al. 2013a), but in the case of *C. obsoletus* and *C. lupicaris* the high variability of the amount of some chemicals may be also attributed to the uncertain age of the specimens and other

factors, such as field variables (trapping dates, breeding sites) and/or methodological variables (temperature conditions, handling and time of washing…).

Cuticular hydrocarbons have been reported as important constituents of the insect surface in dipterans and known to function as species and sex recognition cues, pheromones, allomones and kairomones (Blomquist et al. 1993). In mosquitoes, cuticular hydrocarbons may act as pheromones modulating their mating behaviour. For instance, extracts of *Culex quinquefasciatus*, *C. tarsalis*, and *C. pipiens* resulted attractants for conspecific females (Gjullin et al. 1967) and a contact pheromone from the legs of *Culiseta inornata* allowed males to recognize conspecific females (Lang, 1977). In *Anopheles gambiae*, the proportions of the cuticular hydrocarbons *n*-heneicosane and *n*-tricosane were significantly reduced when the female aged and after it mated (Polerstock et al. 2002). Also, changes in the proportions of *n*-heptadecane, *n*-pentacosane and *n*-hexacosane in the cuticle of female *A. aegypti* were noticed after mating (Polerstock et al. 2002). In midges, only in C. *nubeculosus* a female-produced sex pheromone has been identified as *n*-heptadecane (Mordue et al. 2002). Extracts of volatiles of emerging mixed adults containing the pheromone with sheep blood odours significantly increased the number of matings. In *C. melleus*, extraction of freshly-killed males and females yielded a possible contact mating stimulant pheromone, which elicited significant male response at 1 female equivalent (Linley and Carlson, 1978). The active component was not characterized although several methyl-substituted alkanes produced significant responses but at high concentrations (1 μg). Similarly, a non-identified volatile pheromone produced by swarming males of *A. aegypti* has been reported to stimulate the flying activity of females (Cabrera and Jaffe, 2007). The authors claim that males and possibly also females produce an aggregation pheromone that attracts both sexes towards the swarm.

Our results are consistent with those reported by Linley and Carlson (1982) on the cuticular profile of *Culicoides variipennis* and *C. melleus*. The authors found linear long chain (C21-C33) hydrocarbons as the predominant compounds, but also methyl-branched paraffins of C35-C37 primarily at 11, 13 and 17 positions, and dimethyl-substituted compounds of similar chain length. However, they did not detect the presence of any trimethylalkane. Shorter hydrocarbons (C14-C16) were also noticed, although in minor amounts, in the cuticle of *C. nubeculosus* (González, unpublished). Other studies carried out on Culicidae of the genera *Anopheles* (f.i. *A. aegypti*, *A. gambiae*, *A. hendersoni*, *A. stephensi* and *A. triseriatus*) and *Aedes* (f.i. *A. aegypti*) have reported the predominant

presence of hydrocarbons in a wide range of chain length (C10-C47) (Pappas et al. 1994; Polerstock et al. 2002; Horne and Priestman, 2002; Caputo et al. 2005; Nikbakhtzadeh et al. 2012).

The presence of FAs in the cuticle of Culicidae spp. has been also noticed (Sushchik et al. 2013). Our FAs profile is quite consistent with that of the Asian *Culicoides* (*C. obsoletus*, *C. sinanoensis*, *C. nujiangensis*, *C. punctatus* and *C. pulicaris*) biting midges reported (Jin-Hua et al. 1994). In their study, carried out only by GC, the authors found 15 FAs from which only 10 were identified. FAs C16:0, C16:1, C18:0, and C18:1 were predominant whereas FAs 12:0, 14:0, 18:2, 20:4, 20:0, and 22:0 were present in small-medium amounts. Many of the identified compounds agree with those reported here but in our case FAs 20:0 and 22:0 were not detected in either cuticular nor internal extract. FA 12:0 was detected in trace amounts but discarded in table 1.

The presence of unsaturated FAs with 1, 2 or 3 double bonds has been also frequently reported in insect's epicuticle (Gołębiowski et al. 2008, 2013a; Sushchik et al. 2013) and can serve as precursors to prostaglandins and other insect specific chemicals, such as sex pheromones and cuticular hydrocarbons (Blomquist et al. 1991).

Short- and medium-chain FAs from the curticle have been found to display antimicrobial and fungicidal properties (Sosa-Gomez et al. 1997; Lord and Howard, 2004; Gołębiowski et al. 2013b). Thus, f.i. the toxic effects of FAs 6:0, 7:0, 9:0, 10:0, 18:2 and 18:3 were demonstrated on the conidia of the fungi *Entomophthora culicis*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* (Kerwin, 1982). Determination of the composition of cuticular lipids and their impact on the development and pathogenicity of parasitic fungi may have practical importance because it may allow these fungi or its metabolites to be used in pest control (Gołębiowski et al. 2012b). In fact, entomopathogenic fungi as biological control agents for livestock disease vectors have been active against adult and biting midge larvae of *Culicoides* spp., such as *C. nubeculosus* (Unkles et al. 2004; Ansari et al. 2011).

Fatty esters are involved in brood recognition and have been demonstrated to act as pheromones or kairomones in social and non social insects (Pianaro et al. 2009). Several straight chain C12-C20 esters have been identified in most internal tissues of the biting midges studied. Interestingly, the examined tissues in *F. bipunctata* lack the presence of

these esters almost entirely. The majority of these compounds are methyl, ethyl or isopropyl esters but a couple of acetates were also detected although only in the internal tissues of *C. kibunensis* and *C. fascipennis*. The role of these esters on *Culicoides* spp. is unknown and only in particular cases the biological functions of similar wax esters have been reported, f.i. as pheromones in *Agriotes* spp. (Coleoptera: Elateridae) (Tóth et al. 2002).

Aldehydes are not commonly known components of insect cuticular lipids (Buckner, 1993), and, thus, in our case only long chain aldehydes (C20-C30) have been detected in moderate amounts in the cuticle of *C. lupicaris*. Fungistatic properties have been attributed to aldehydes in some insect species (Sosa-Gomez et al. 1997; Howard and Lord, 2003) but none of them on mosquitoes.

Cholesterol and its precursor squalene were detected in high amounts, predominantly in internal tissues in almost all biting midges studied, in agreement to other studies on the blow-fly *Lucilia sericata* (Gołebiowski et al. 2012b). In blood-feeders arthropods, cholesterol is very abundant in *A. gambiae* cuticle (Caputo et al. 2005) but sterols are also common in assassin bugs (Juarez and Blomquist, 1993) and ticks (Yoder et al. 1993). Here, again, both chemicals were alsmost completely absent in the non-biting midge *F. bipunctata*.

Assuming that most *Culicoides* females are anautogenous insects, requiring, therefore, blood meal to develop eggs, sterols are particularly necessary for egg production and normal embryogenic development. In this context, Dyer et al. (2008) reported that in the first gonotrophic cycle of gravid mosquitoes more than half of blood meal-derived FAs and cholesterol is deposited into eggs.

With regard to alcohols and lactones, to our knowledge neither alcohols nor isocoumarins have been reported previously in any *Culicoides* spp. However, we have noticed the presence of 1-hexadecanol in both types of extracts of *C. obsoletus* and in the cuticle of *C. lupicaris* although in small amounts (0.1-1%). The amounts of the alcohol varied considerably, even in specimens with the same gonotrophic stage, from a maximum of 1.26 ± 0.21 ng per insect to traces. The non-biting midge *F. bipunctata* contained 1-5% of isocoumarin in both cuticular and internal tissues. Diverse biological functions of cuticular alcohols and lactones have been reported mainly in Hymenoptera (Arn et al.

1998; Donze et al. 1998) and in the behaviour of *Anastrepha* fruit flies (Lima et al. 2001), respectively, but remains unknown in blow flies (Gołębiowski et al. 2012b).

One important aspect in epidemiological studies of vector borne biting midges is to estimate the age of wild populations, particularly females. Knowledge of the age of the midges is important not only to evaluate population changes over time and to draw conclusions about mortality and fecundity rates, but also to provide information about the timing of transmission of potential vectors and the risk and implications of spreading virus-mediated diseases (Hugo et al. 2006). Determination of the age would also be important to build models for age grading of natural specimens, as it has been shown for other Culicidae of the genus *Aedes* (Desena et al. 1999; Gerade et al. 2004), *Anopheles* (Brei et al. 2004; Nikbakhtzadeh et al. 2012) or *Culex* (Chen et al. 1990).

We have tried to correlate the estimated age of *C. obsoletus* and *C. lupicaris* of different gonotrophic stages (nutritional status) with the amount of the major compounds present in their cuticular and internal extracts. In our study, the first gonotrophic stage (named "non-pigmented") includes young individuals with a non-pigmented abdomen. After feeding blood, which midges require for egg development, the abdomen becomes red ("blood-engorged" stage). Maturation of eggs require approx. 7-10 days depending on species and temperature, and this corresponds to the period determined for the BTV incubation in *Culicoides* to become transmissible to a susceptible host (Carpenter et al. 2011). After this time, the abdomen is filled with eggs and this stage ("gravid") is linked with BTV-induced high infection rates (Savini et al. 2004). After oviposition, the abdomen becomes purple ("pigmented stage") and then females are in the last stage of their life to harbour and transmit the virus from their salivary glands (Romón et al. 2009). Unfortunately, no significant differences were obtained in the amount of most of the compounds considered perhaps because of the different ages and development sites of the wild specimens considered. Only the amounts of cholesterol and squalene in gravid and blood-engorged females appear to be significantly higher than the corresponding values in non-pigmented and pigmented individuals.

In relation to cluster tree based on all chemicals profile showed little similarity and opposed interpretations of similarity. In part, it is in accordance with the fact that studied species belong to four different subgenera: *C.* (*Avaritia*) *obsoletus*; *C.* (*Culicoides*) *lupicaris*; *C.* (*Oecacta*) *kibunensis; C.* (*Silvaticulicoides*) *fascipennis*. It is important to take

into consideration that closely phylogenetic relationships do not involve having similar compounds (Martin and Drijfhout, 2009). Both studied genera are better separated in internal tissues than in cuticular extracts. A reasonable explication could be the different feeding habits (i.e. *Culicoides* feed on mammal's blood and occasionally feed on sugar sources and *Forcipomyia bipunctata* sucks insects hemolymph). In the same way, the use of chemical markers (hydrocarbons or others) with taxonomical purposes must be taken into account as an additional tool for discerning *Culicoides* cryptic species, in particular *Culicoides obsoletus*/*Culicoides scoticus* sibling species.

Table 1. List of compounds tentatively identified in cuticular and internal extracts of *Culicoides* spp. and *Forcipomyia bipunctata* females [a]

				C. obsoletus ♀♀		*C. lupicaris* ♀♀		*C. kibunensis* ♀♀		*C. fascipennis* ♀♀		*F. bipunctata* ♀♀	
Peak	**ID**	**MW**	**Compound**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**
		Hydrocarbons											
1	S/CI/L	212	*n*-Pentadecane C15	-	-	-	-	-	-	-	-	+	t
2	S/CI/L	238	*n*-Heptadecene [b]	-	-	-	-	-	-	-	-	+	-
3	S/CI/L	240	*n*-Heptadecane C17	+++	+	++	+	++	+	+	+	++	+
4	S/CI/L	254	*n*-Octadecane C18	-	-	-	t	-	-	-	-	-	-
5	S/CI/L	268	*n*-Nonadecane C19	++	-	+	+	+	+	+	-	+	t
6	S/CI/L	296	*n*-Henicosane C21	t	t	t	t	-	-	+	-	-	-
7	S/CI/L	324	*n*-Tricosane C23	++	t	++	+	++	-	+++	-	+	-
8	S/CI/L	352	*n*-Pentacosane C25	++	++	++	+	++	++	+	++	++	t
9	S/CI/L	366	*n*-Hexacosane C26	-	-	+	-	-	-	t	-	t	-
10	S/CI/L	380	*n*-Heptacosane C27	++	++	++	+	++	++	++	++	++	+
11	S/CI/L	394	*n*-Octacosane C28	-	-	+	-	-	-	t	-	-	-
12	S/CI/L	408	*n*-Nonacosane C29	++	++	++	+	++	+++	+	++	+	t
13	S/CI/L	422	11-/13-/15-MeC29 [c]	-	-	-	-	-	-	-	-	++	-
14	S/CI/L	436	11,13-DiMeC29 + 13,15-DiMeC29 [d]	-	-	-	-	-	-	-	-	++	-
15	S/CI/L	436	Hentriacontane C31	+	-	-	-	-	-	-	-	-	-
16	S/CI/L	464	Tritriacontane C33	++	-	-	-	t	-	t	-	-	-
17	L	492	15,19-DiMeC33	-	-	-	-	++	-	-	-	-	-
18	L	506	15,18-DiMeC34	-	-	-	-	-	-	++	-	-	-
19	CI/L	506	13-/15-/17-MeC35 [c]	+++	t	+	-	++	-	++++	-	+	-
20	CI/L	520	15,19-DiMeC35	-	-	-	-	-	+	-	-	-	-
21	CI/L	520	11,14-DiMeC35 + 13,16-DiMeC35	++++	t	+++	t	++++	-	++	-	+	-
22	CI/L	534	11,15,19-TriMeC35	t	t	++	t	-	-	++	-	-	-

				C. obsoletus ♀♀		*C. lupicaris* ♀♀		*C. kibunensis* ♀♀		*C. fascipennis* ♀♀		*F. bipunctata*♀♀	
Peak	**Source**	**MW**	**Compound** [b]	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**
23	CI/L	534	13,17,21-TriMeC35	-	-	-	-	+++	-	-	-	-	-
24	CI/L	548	11-/13-MeC38 [c, d]	-	-	+	-	++	-	+	-	-	-
25	L	548	MeC38 mixture [d]	-	-	+	-	++	-	-	-	-	-
			Fatty acids										
26	CI/L	116	Hexanoic acid	-	-	-	-	-	-	-	+	-	-
27	L	144	2-Ethyl hexanoic acid	-	t	-	t	-	t	t	+	-	-
28	CI/L	144	Octanoic acid	-	t	-	+	-	t	-	+	t	t
29	CI/L	158	Nonanoic acid	+	+	+	+	+	+	+	++	+	t
30	CI/L	172	Decanoic acid	-	t	-	t	-	t	-	-	-	-
31	L	228	Tetradecanoic acid	-	-	t	+	++	++	-	+	+	-
32	CI/L	254	Hexadecenoic acid [b]	+++	++++	++	++++	++	++++	++++	++++	+++++	++
33	S/CI/L	256	Hexadecanoic acid	++	+++	+++	++++	++	+++	++++	++++	+++	++
34	L	268	Heptadecenoic acid [b]	t	-	t	-	-	-	-	-	-	-
35	S/CI/L	280 282	C18:2 + C18:1 Linoleic + oleic acid	+++	+++++	++++	+++++	++	+++++	+++	+++++	+++++	++++
36	CI/L	284	Octadecanoic acid	-	+	+	+++	+	+	+	++	+	+
37	CI/L	306	Arachidonic acid + dihydroarachidonic acid [b, d]	+	-	++	-	t	-	++	-	++	-
			Esters										
38	L	242	iso-Propyl dodecanoate	-	-	-	t	-	-	-	+	-	-
39	L	270	iso-Propyl tetradecanoate	+	++	t	++	t	++	t	++	-	-
40	L	268	Metyl 9-hexadecenoate	-	-	-	-	-	-	-	-	-	+
41	L	284	Ethyl hexadecanoate	-	++	-	t	-	-	-	+	-	-
42	L	298	iso-Propyl hexadecanoate	+	++	+	++	-	++	t	+++	-	-
43	L	312	Isooctyl dodecanoate [d]	+	t	-	-	-	-	-	t	-	-
44	L	310	Ethyl octadecenoate [b]	-	++	-	-	-	-	-	-	-	-
45	L	312	*n*-Octadecyl acetate	-	-	-	t	-	-	-	++	-	-
46	L	340	*n*-Eicosanyl acetate	-	-	-	t	-	++	-	++	-	-

				C. obsoletus ♀♀		*C. lupicaris* ♀♀		*C. kibunensis* ♀♀		*C. fascipennis* ♀♀		*F. bipunctata* ♀♀	
Peak	**Source**	**MW**	**Compound**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**	**Cuticular**	**Internal**
			Aldehydes										
47	CI/L	142	Nonanal	t	-	-	t	t	+	+	t	-	-
48	CI/L	156	Decanal	t	-	t	t	t	t	+	+	-	-
49	CI/L	296	Icosanal	-	-	+	t	-	-	-	-	-	-
50	CI/L	324	Docosanal	-	-	++	-	-	-	-	-	-	-
51	S/CI/L	352	Tetracosanal	t	-	++	-	-	-	-	-	-	-
52	CI/L	380	Hexacosanal	-	-	+	-	-	-	-	-	-	-
53	CI/L	408	Octacosanal	-	-	++	-	t	-	-	-	-	-
54	CI/L	436	Triacontanal	-	t	+	-	t	-	-	-	-	-
			Terpenes										
55	L	194	Geranyl acetone	t	t	-	t	t	t	t	+	t	t
56	CI/L	333	Geranyl-geraniol acetate [d]	-	-	-	-	+	-	-	t	+	+++++
57	S/CI/L	411	Squalene	++	+++++	+	+	+	++	t	++++	-	t
58	L	-	Terpenic alcohol [d]	-	-	-	++	-	+	-	+++	++	+++++
			Steroids										
59	S/CI/L	387	Cholesterol	+++++	+++++	+	++++	+++	+++++	+++	+++++	+	-
			Alcohols										
60	S/L	242	1-Hexadecanol	+	+	+	-	-	-	-	-	-	-
			Lactones										
61	CI/L	178	Isocoumarin	-	-	-	-	-	-	-	-	++	++
			Unknown compounds										-
62	L	422	Unknown 1	-	-	++	-	-	-	++	-	+	-
63	L	-	Unknown 2	-	-	-	-	-	-	-	-	-	+++++

Symbols represent the percentage of each compound relative to the total abundance: t (traces): ≤0.1%; +: 0.1-1%; ++: 1-5%; +++: 5-10%; ++++:10-20%; +++++; ≥ 20%.

ID: Identifying source; MW: Molecular weight; S: Standard; CI: Chemical ionization; L: Literature.

[b] Position of the unsaturation was not determined.

[c] A dash followed by a forward slash (-/) between numbers refers to mixture of isomers.

[d] Tentatively identified.

7. CONCLUSIONS

In conclusion, we have provided new data of the chemical characterization of the cuticle and internal extracts of wild species of biting midges (*Culicoides* spp.) in comparison to a non-biting midge (*Forcipomyia bipunctata*) with remarkable differences between both groups. Our results provide a first step for further studies on the possible implication of the identified chemicals in the behaviour and intraspecific communication of these hematophagous insects and as additional tool for taxonomic studies.

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FINAL CONCLUSIONS

❖ CHAPTER 1-2

1. It is reported the presence of a total of 52 *Culicoides* species distributed throughout the Autonomous Community of the Basque Country (48 livestock-associated and 28 wildlife-associated) of which, four were new records to the Iberian Peninsula: *C. heliophilus, C. paradisionensis, C. clastrieri* and *C. tauricus.*

2. The most common and spatially well-distributed species over the sheep sampling sites is the complex Obsoletus which comprises *Culicoides obsoletus* and *C. scoticus*, both accounted for between 70 to 90% of the total collections depending on the sampled farms. *Culicoides lupicaris* is highlighted as the second most abundant species. In contrast, in natural habitats the most common species are *C. festivipennis, C. alazanicus* and *C. brunnicans* depending on the ecotone.

3. The distribution of *C. imicola* in the Basque Country, is rare, with only eight captures linked to sheep farms in the Oiartzualdea valley (Gipuzkoa, where the first outbreaks of the BTV occurred), a single specimen captured in Biscay and none in Alava.

4. In sheep farms, *Culicoides* populations are active between April and November but peak particularly in the summer months (June, July and August) depending on localities and age studied. In some farms a second population peak of *Culicoides* can be observed in the months of October and November, linked with the previous rains ocurred followed by a short time period of moderate temperatures. In contrast, in natural ecosystems, May and September were the months with more collections.

5. The activity of *Culicoides* midges during winter months is related to the minimum nocturnal temperatures. While *Culicoides* midges were present throughout the year and even during the winter in the provinces of Gipuzkoa and Biscay (Atlantic area temperatures), no catches occurred in winter at the southern farms of Alava, where the night temperatures are much colder in that season and fluctuate near zero degrees. Therefore, there is likely to be a periood of two-three months (January, February and March) which are free of *Culicoides* vectors in certain localities of Alava.

6. *Culicoides* midges follow an emergence pattern linked to two particular factors: precipitation and temperature pattern. *Culicoides brunnicans* and *C. picturatus* are specially abundant immediately after the spring rains have ended (April-May), shortly after, other species such as *C. simulator, C. lupicaris*/*C. pulicaris* and the subgenus *Silvaticulicoides* also appear in moderate numbers. After a period of various days with warm temperatures and climate stability, the complex Obsoletus (*C. obsoletus*/*C. scoticus*) emergences in high numbers. The species compositon of these midges suffer important changes due to the influence of different factors both anthropic and climatic (likely to be more remarkable in recent years due to the existence of a widely recognized potential climate change). The rainfall patterns are directly related to the creation of oviposition areas with humidity, which serve as breeding sites for midges, and therefore summer months with low precipitations, as well as changes in manure management and grazing can have substantial implications on the *Culicoides* ecology. This can result in a important changes in the number and phenology of biting midges from one year to the next in the same farm.

❖ CHAPTER 3

7. The developmental sites of 28 *Culicoides* species have been described and identified. It comprises a total of 12 micro-habitats of moist and semi-acuatic substrates linked both to livestock activity and suitable substrates on the farm environment. Other five micro-habitats resulted infertile for *Culicoides*. There is an important influence of the humidity gradient in the horizontal distribution of *Culicoides* midges at pond habitat, 50% of the specimens emerged from mud at the water edge, as opposed to 50 cm above (32%) and 1 meter above waterline (18%). The majority of the specimens were found in the upper layer (0-3 cm). In manure, larvae preferred deeper layers (≥6 cm).

8. Potential key vectors of veterinary interest were found in two main terrestrial types of micro-habitats, which are poor in number of species but for accounted most of the specimens:

a) *Culicoides obsoletus* was found in the three types of manure (69% of the total *Culicoides* spp. collections), the old and composted manure stored as heap manure outdoors (61% of the total collections of this species), manure mixed with organic matter (28%) and fresh manure (11%).

b) Larvae of *C. scoticus* (94% of the total emergences of this species) and *C. lupicaris* (37%) are associated to edaphic susbtrates with rotting leaf litter situated in dark and wet areas that included the parasitic plant *Lathraea clandestina.*

9. The grass ditch (runoff area) which receives the water from the barn roof and the four micro-habitats associated with the pond are the richest in number of species.

10. In pond micro-habitats, the most common species are *C. festivipennis, C. punctatus, C. brunnicans* and *C. circumscriptus,* in river edges is *C. kibunensis,* in the poplar grove is *C. brunnicans,* in the roof runoff area is *C. punctatus* species and in the forest mud is *C. lupicaris.*

11. The surveillance, monitoring and compliance of a set of simple, clear and mitigating actions by farm owners could be important measures to impede the rearing of adult *Culicoides* avoiding massive breeding. It is worth highlighting that the use of methods such as composting and/or acidification and/or drying the heaps of manure and/or covering the heaps of manure with canvas could block or reduce biting midge populations. The importance of keeping organic residues clear from farm entrances, avoiding temporary puddles and pools of water around farms and the installation of piped water supply systems etc. is also to be emphasized.

❖ CHAPTER 4

12. None of the evaluated compounds with putative attraction to mosquitoes and biting midges species were especially attractive for *Culicoides obsoletus* with olfactometer assays. None *Culicoides* species were attracted to different doses of 1-octen-3-ol and gaseous carbon dioxide in independient field trials performed with CDC traps (without light sources).

13. The study of 23 repellent formulations in laboratory assays have allowed the behaviour observation of *C. obsoletus* "*in situ*". Some discrepances detected among assays are linked with inherent factors of the perception mechanisms and evaporation rates of the repellents. However, the laboratory studies serve an an important tool as a first screening for discerning between putative repellents.

14. The repellent DEET, the mixture of fatty acids (1:1:1, octanoic, nonanoic and decanoic) and lemon eucalyptus oil substantially reduced the number of captures of *Culicoides* with respect to control traps in assays performed with light traps.

15. At both the tested doses of repellent solution, DEET traps captured between 2-2.2 times (10%) and 2.7-3.3 times (25%) fewer total *Culicoides* spp. than control traps. Fatty acids between 2.2 (10%) and 3.6 times (25%) and the lemon eucalyptus 2.7 times (25%).

16. Within the captured *Culicoides* species, the subgenus *Silvaticulicoides* seems to be more resilient to some of the repellents tested.

❖ CHAPTER 5

17. The chemical profile of four *Culicoides* species were reported, with a total of 67 compounds identified by chromatographic techniques (GC-MS) in cuticular and internal tissues (55 cuticular and 43 internal compounds).

18. The detected compounds belong to seven major chemical classes: fatty acids (C6-C20), with C16:0, C16:1 and C18:1 as dominant, branched hydrocarbons (C29-C38 mono/di/trimethylalkanes), linear hydrocarbons (C15-C33), terpenes (squalene), steroids (cholesterol), aldehydes (C20- C30), esters and other minor constituents. The relative contents of linear and branched hydrocarbons, and aldehydes were high in cuticular extracts but practically absent in internal tissues. In contrast, fatty acids, terpenes and steroids were, in general, high in internal tissues.

19. No volatile compounds have been found in *Culicoides obsoletus* specimens with the techniques used (absorbent cartridges and SPME fiber) in laboratory assays.

CONCLUSIONES FINALES

❖ CAPITULO 1-2

1. Se han identificado un total de 52 especies de *Culicoides* distribuidas a lo largo de la Comunidad Autónoma del País Vasco (48 de ellas asociadas a explotaciones de ganado ovino y 28 a hábitats naturales), siendo cuatro de ella nuevas citas para la Península Ibérica: *C. heliophilus, C. paradisionensis, C. clastrieri* y *C. tauricus*.

2. El grupo de especies más abundante y mejor distribuidas geográficamente en las granjas de ovino es el complejo Obsoletus formado por *Culicoides obsoletus* y *C. scoticus*, ambas especies comprenden entre el 70 al 90% del total de capturas según provincias muestreadas. En segundo lugar cabe destacar la presencia de *C. lupicaris* como la más abundante. En cambio, en los hábitats naturales las especies más comunes son *C. festivipennis, C. alazanicus* y *C. brunnicans*, dependiendo del tipo de ecotono.

3. La presencia de *Culicoides imicola* en el País Vasco, es excepcional, con capturas muy puntuales, sólo ocho individuos ligados a los primeros brotes de LA en las granjas del valle de Oiartzualdea en Guipúzcoa, un único ejemplar en Vizcaya y ninguno en el territorio de Álava.

4. En las granjas de ovino, las poblaciones de *Culicoides* ejercen su actividad entre abril y noviembre, siendo especialmente abundantes en los meses centrales del verano (junio, julio y agosto) según localidades y año estudiados. En algunas granjas puede observarse un segundo pico de población de *Culicoides* en los meses de octubre y noviembre, vinculados muy probablemente a las lluvias acontecidas seguidas de un corto periodo de tiempo con temperaturas moderadas. En cambio en los ecosistemas naturales, mayo y septiembre son los meses con más capturas.

5. La actividad de los *Culicoides* durante los meses de invierno está relacionada con las temperaturas mínimas nocturnas. Mientras que en las provincias de Guipúzcoa y Vizcaya se capturan un número de ejemplares mínimo durante los meses más fríos invernales, estos no se capturan en algunas granjas de Álava donde las temperaturas nocturnas fluctúan entorno a ± 0º C. Por ello, es factible que exista un periodo de unos dos o tres meses (diciembre, enero, febrero) libres de vectores en ciertas localidades alavesas.

6. Los *Culicoides* siguen un patrón de emergencia ligado especialmente al régimen de precipitaciones y de temperatura. *Culicoides brunnicans* y *C. picturatus* son muy abundantes tras los días siguientes a las lluvias primaverales sucedidas en abril-mayo, seguidamente aparece *C. simulator*, *C. lupicaris*/*C. pulicaris* y el subgénero *Silvaticulicoides*. Tras un periodo de varios días de temperaturas cálidas y estabilidad hace su presencia masiva el grupo Obsoletus (*C. obsoletus*/*C. scoticus*). La composición de especies de estos mosquitos sufre importantes cambios debido a la influencia de diferentes factores tanto antrópicos como climáticos (probablemente más acusados en los últimos años por la posible existencia de un cambio climático). El régimen de lluvias está directamente ligado a la creación de medios de ovoposición que aportan la humedad necesaria para la cría de estos mosquitos, por ello la escasez de precipitaciones durante el verano así como los cambios en el manejo del estiércol y pastoreo pueden suponer importantes desajustes ecológicos que se manifiestan con importantes cambios en el número y fenología estos mosquitos de un año a otro en la misma granja estudiada.

❖ CAPITULO 3

7. Los hábitats de cría de 28 especies del género *Culicoides* han sido descritos e identificados y comprenden un total de 12 micro-hábitats húmedos y/o semi-acuáticos ligados tanto a medios derivados de la actividad ganadera como a sustratos presentes en el propio entorno de la granja. De otros cinco hábitats no se obtuvieron ejemplares. Se aprecia una importante influencia del gradiente de humedad en la distribución horizontal de los *Culicoides* en el hábitat de la charca, encontrándose menos individuos a medida que nos alejamos perpendicularmente del borde de agua de la charca (borde: 50% de los ejemplares, a 50 cm 32% y a 1 m 18%) y se distribuyen particularmente en las capas más superficiales (0-3 cm). En el caso del estiércol las larvas habitan en capas más profundas (≥6 cm).

8. Se destacan particularmente dos hábitats por contener las especies de mayor interés veterinario. Estos hábitats contienen el grueso de ejemplares aún siendo pobres en número de especies:

a) El estiércol en sus tres formas alberga el 69% de todas las capturas de *Culicoides*, en concreto, el hábitat preferido de cría para *C. obsoletus s.s.* es el estiércol maduro almacenado en montones en el exterior del establo (61% del total de capturas de esta

especie), la materia orgánica con estiércol mezclado en la esquina de la entrada al establo (28%) y el estiércol fresco (11%).

b) Sustratos edáficos húmedos y sombríos en las proximidades de las instalaciones ganaderas donde se acumula hojarasca en descomposición y crece la planta *Lathraea clandestina* es el hábitat elegido por *Culicoides scoticus* (94% de sus emergencias) y *C. lupicaris* (37%).

9. La zanja herbácea que recibe el agua de goteo procedente del tejado del establo así como los cuatro micro-hábitats asociados con la charca son los más ricos en especies.

10. En los micro-hábitats de la charca y su entorno próximo, las especies más frecuentes son *C. festivipennis*, *C. punctatus*, *C. brunnicans* y *C. circumscriptus*, en los márgenes del río *C. kibunensis*, en la chopera *C. brunnicans*, en la zanja de goteo *C. punctatus* y en el barro del monte próximo a la granja lo es *C. lupicaris.*

11. Se plantean una serie de medidas preventivas y paliativas para reducir, impedir y/o desfavorecer los medios donde se produce la cría masiva de los *Culicoides*. Se destaca el uso de compostadoras para el secado del estiércol y/o acidificación y/o tapar los montones de estiércol con lonas impidiendo o reduciendo el acceso de los mosquitos y de la humedad. Evitar la formación de charcos que permanezcan anegados durante cierto tiempo, limpiar los residuos orgánicos almacenados en las entradas del establo, instalación de sistemas de canalizado del agua de la lluvia en el tejado etc.

❖ CAPITULO 4

12. Ninguno de los compuestos evaluados con supuesto potencial atractivo para algunas especies de mosquitos y de *Culicoides*, fueron especialmente atrayentes para *Culicoides obsoletus* mediante técnicas de olfactometría. Ninguna especie de *Culicoides* se sintió atraida hacia differentes dosis de 1-octen-3-ol y CO_2 gaseoso en pruebas de campo independientes realizadas mediante trampas CDC (sin luz).

13. A partir del estudio de 23 formulaciones de repelentes en ensayos de laboratorio se ha observado el comportamiento de *C. obsoletus* "*in situ*". Se han detectado ciertas discrepancias entre los diferentes ensayos, ligados a diferentes factores inherentes a

mecanismo de percepción y tasas de evaporación de los repelentes. Sin embargo, los estudios de laboratorio tienen su importancia como primera selección para discernir entre los compuestos con efecto más repelente.

14. Los repelentes DEET, la mezcla de ácidos grasos (octanoico, nonanoico y decanoico) en proporción 1:1:1 y el eucalipto de limón reducen considerablemente las capturas de *Culicoides* respecto al control en ensayos llevados a cabo mediante trampas de luz.

15. De las dosis evaluadas, las trampas impregnadas con DEET reducen el número de capturas totales de *Culicoides* entre 2 y 2,2 veces (al 10% de solución repelente) y entre 2.7 y 3,2 veces (al 25%) respecto a trampas control. Los ácidos grasos entre 2,2 (10%) y 3.6 veces (25%) y el eucalipto de limón 2,7 veces (al 25%).

16. Dentro de las especies capturadas, el subgénero *Silvaticulicoides* es el grupo de especies más resistente o menos vulnerable al efecto de los repelentes.

❖ CAPITULO 5

17. El perfil químico tanto cuticular como interno de cuatro especies de *Culicoides* fue identificado mediante técnicas cromatográficas (GC-MS) reportándose un total de 67 compuestos (55 de ellos presentes en la capa cuticular y 43 en tejidos internos).

18. Los compuestos detectados pertenecen preferentemente a siete clases químicas: ácidos grasos (C6-C20), con C16:0, C16:1, C18:1 como dominantes, hidrocarburos ramificados (C29-C38 mono/di/trimetil-alcanos), hidrocarburos lineales (C15-C33), terpenos (escualeno), esteroides (colesterol), aldehídos (C20-C30), ésteres y otros como constituyentes menores. El contenido relativo de hidrocarburos ramificados y aldehídos fue mayor en extractos cuticulares, estando prácticamente ausentes en tejidos internos. Por el contrario, el contenido de ácidos grasos, terpenos y esteroides fue en general mayor en capas internas.

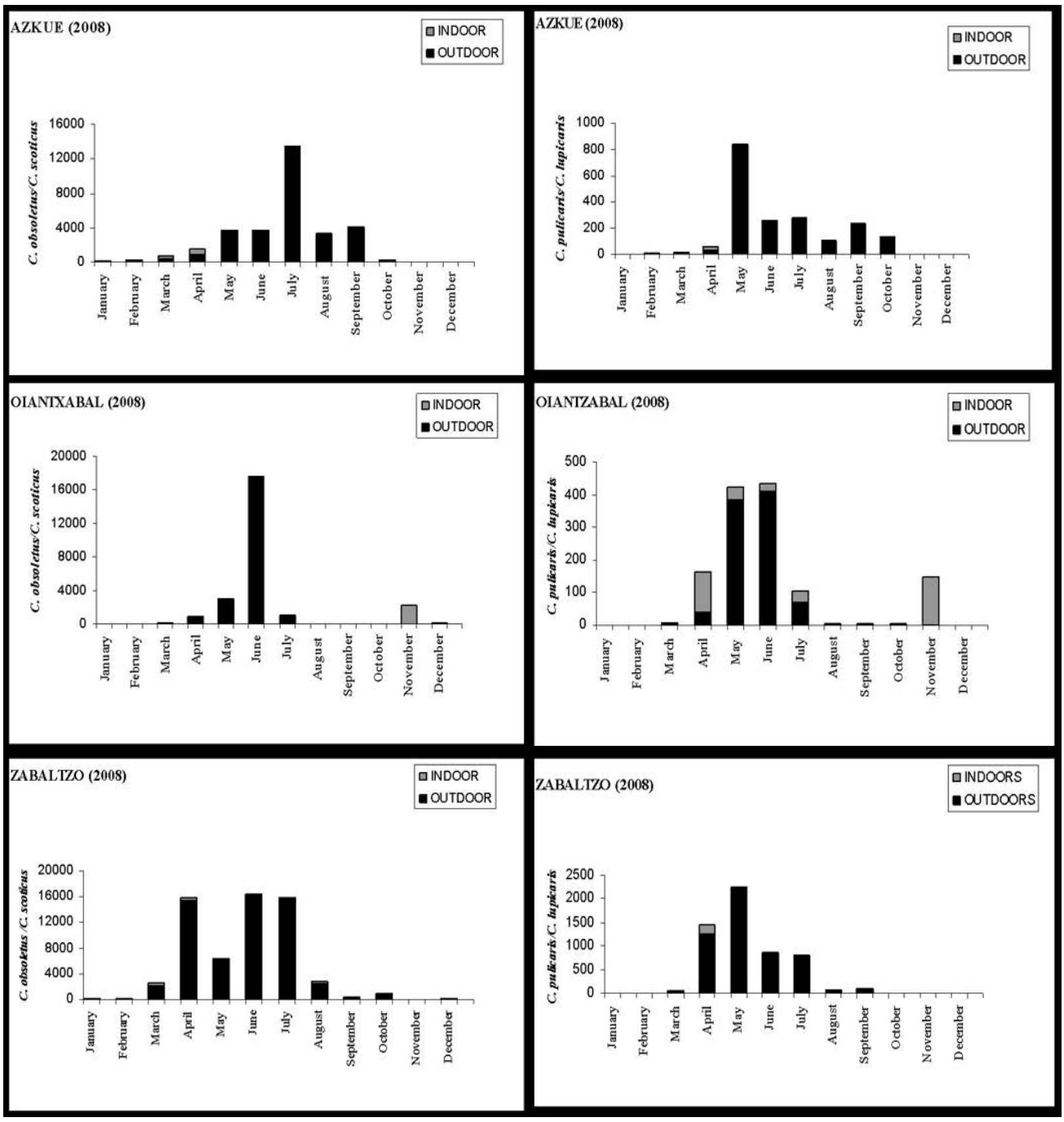
19. No se ha encontrado ningún compuesto de tipo volátil con las técnicas implementadas (tubos de absorción y fibra SPME) en ensayos de laboratorio llevados a cabo con ejemplares de *Culicoides obsoletus.*

APPENDIX

APPENDIX I (CHAPTER 2)

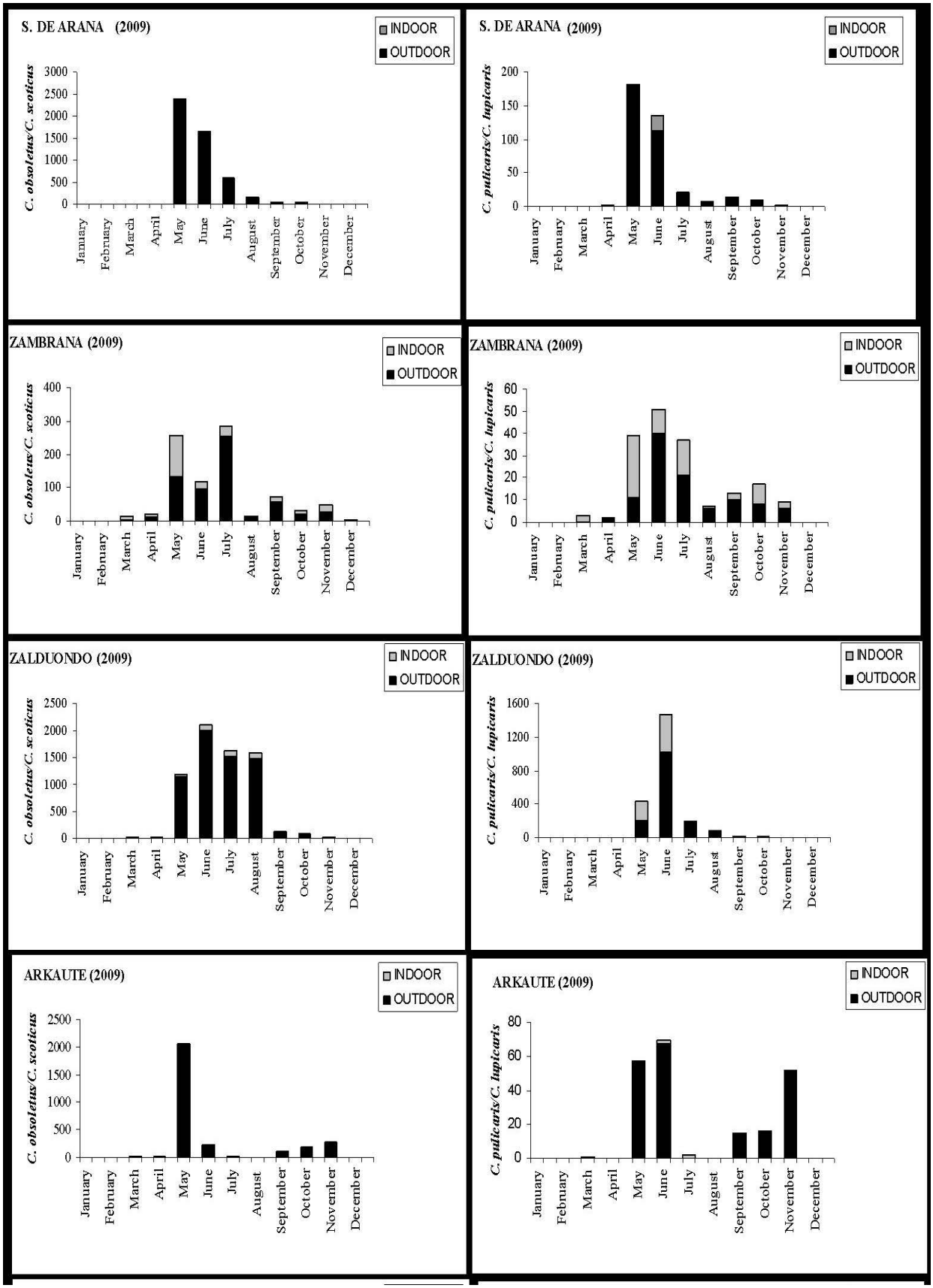
1. FARM SETTINGS

i) *Gipuzkoa sampling settings*



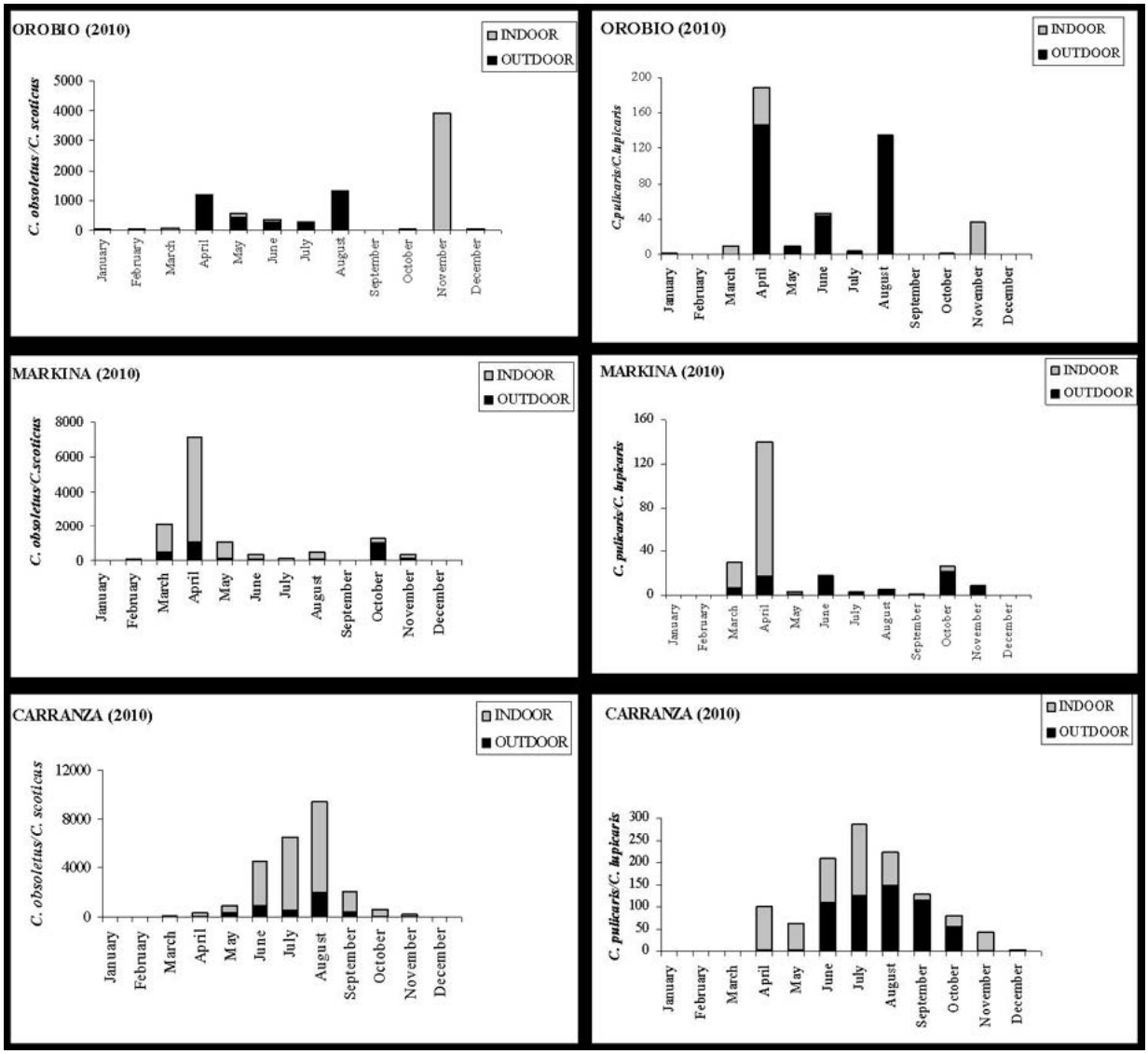
Appendix I (1). Comparative outdoors (black bar) and indoors (grey bar) *Culicoides* relative composition in farms from Gipuzkoa (2008). On the left *C. obsoletus*/*C. scoticus* and on the right *C. pulicaris/C. lupicaris.*

ii) *Alava sampling settings*

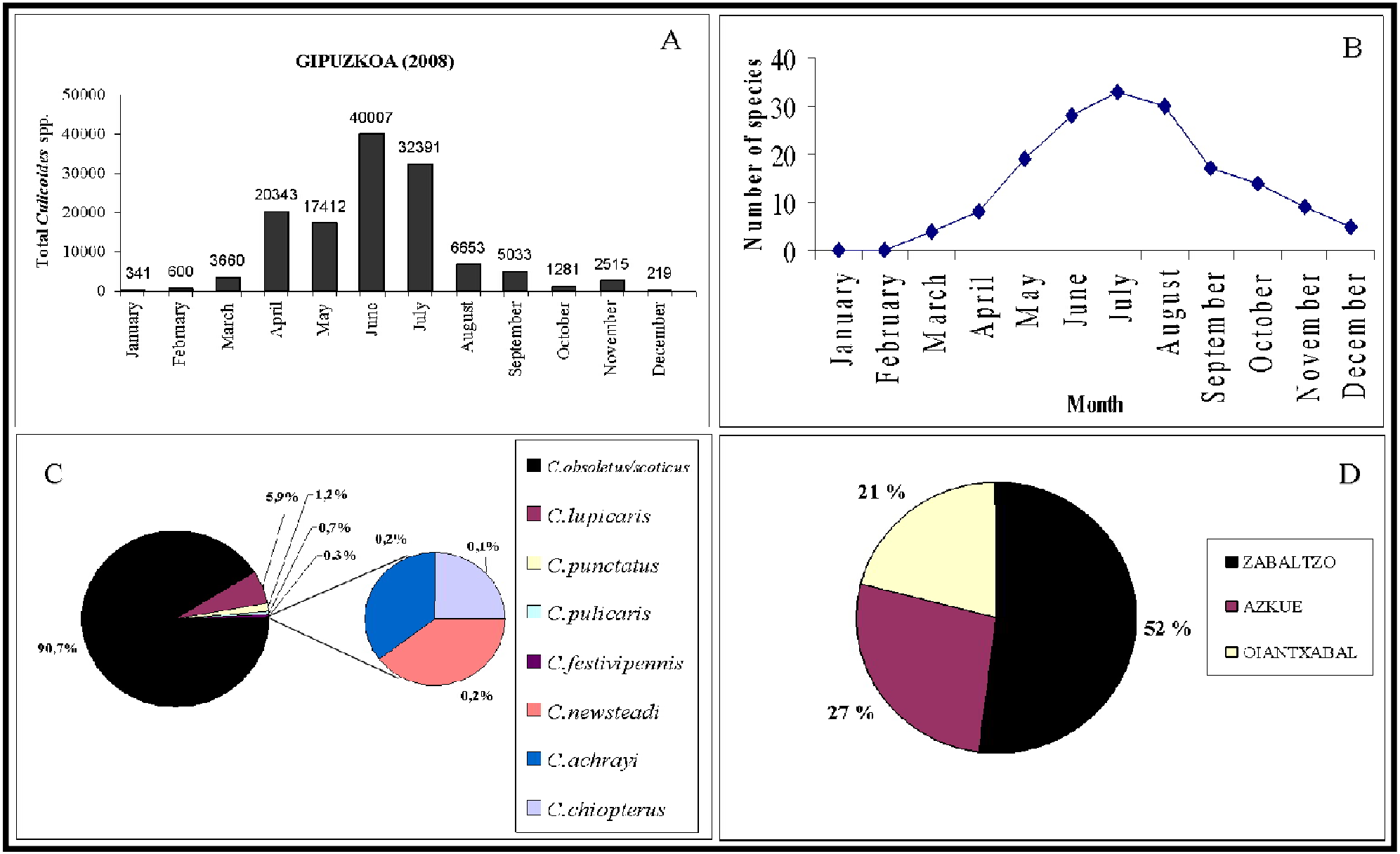


Appendix I (2). Comparative outdoors (black bar) and indoors (grey bar) *Culicoides* relative composition in farms from Alava (2009). On the left *C. obsoletus*/*C. scoticus* and on the right *C. pulicaris/C. lupicaris.*

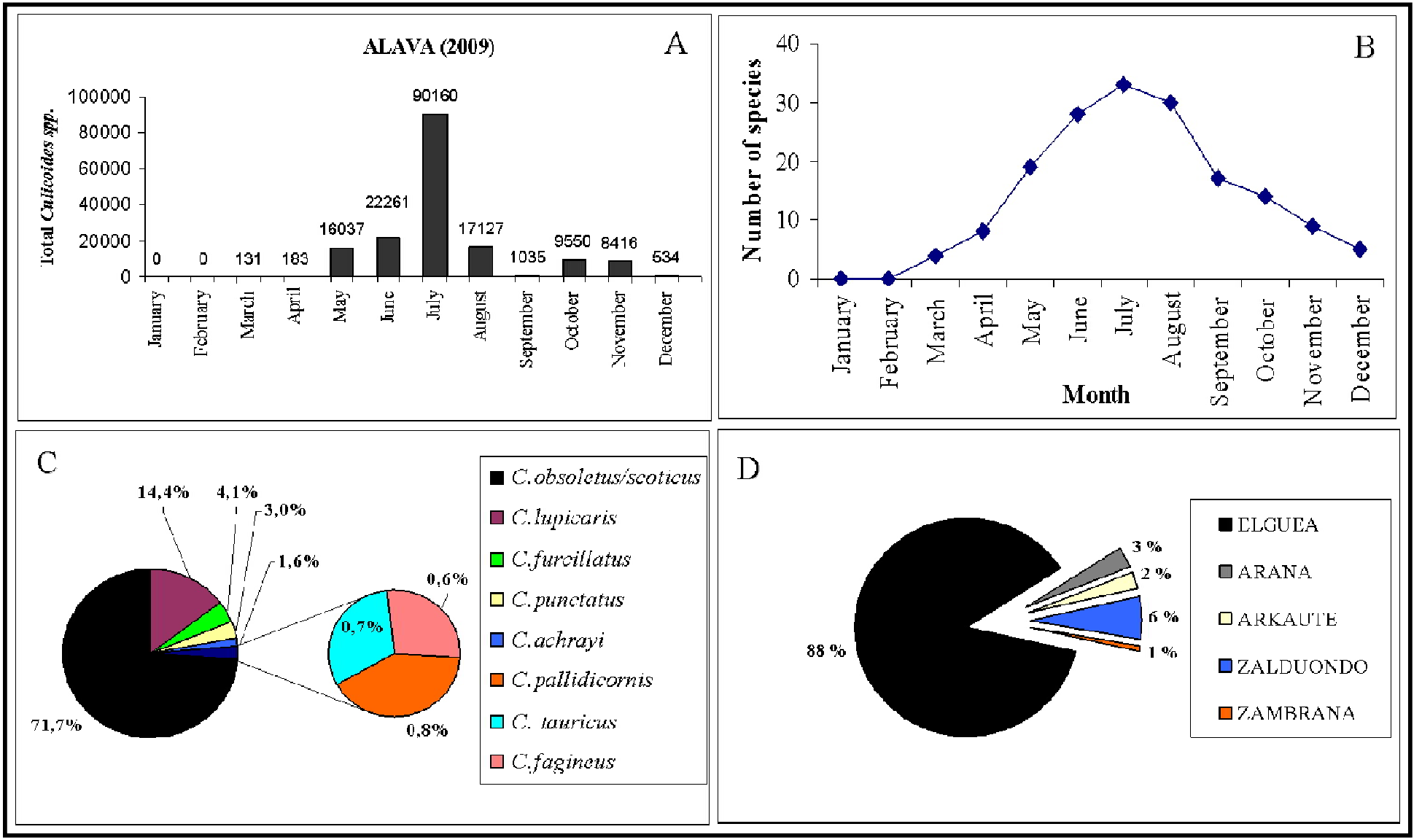
ii) *Biscay sampling settings*



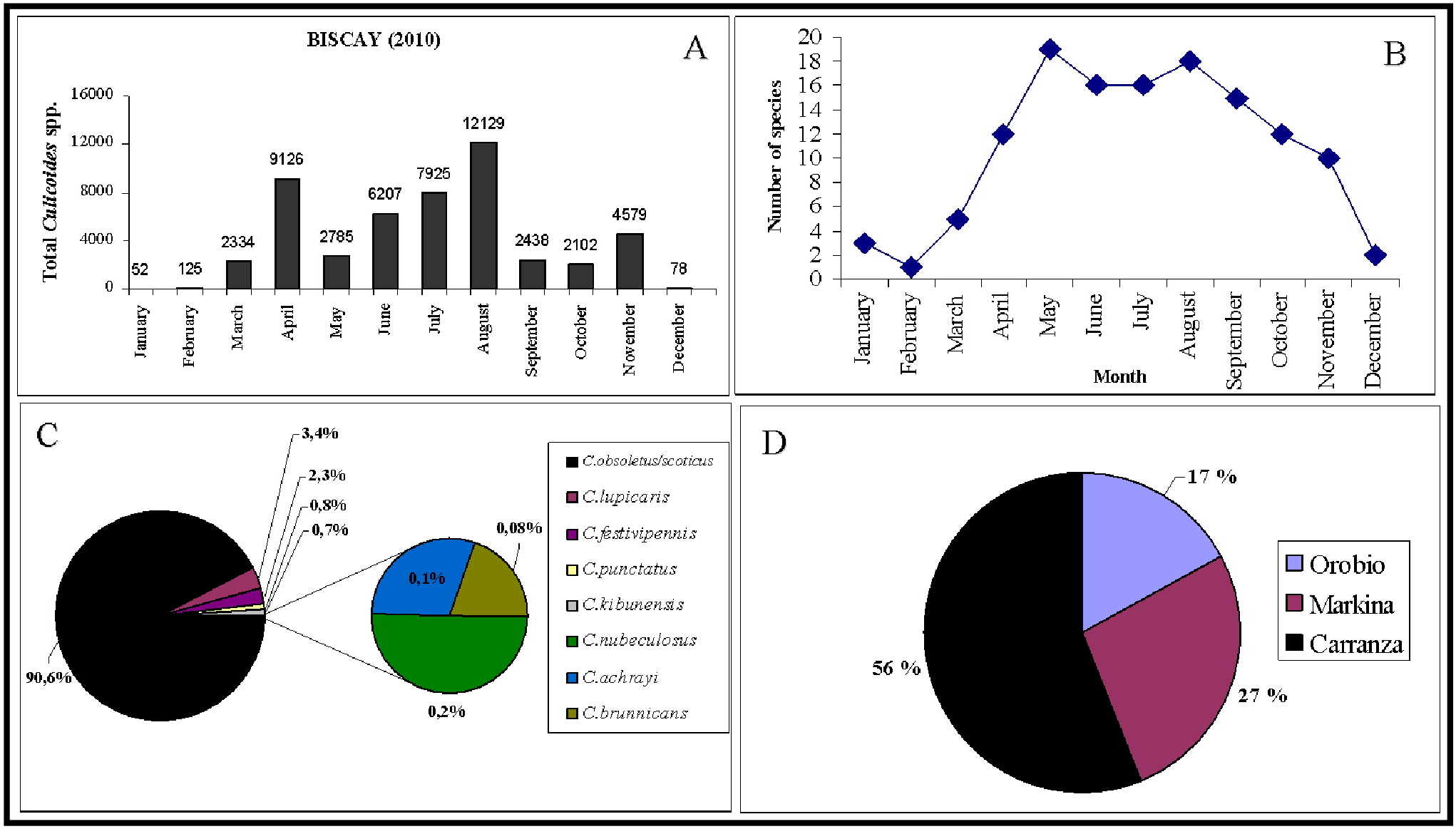
Appendix I (3). Comparative outdoors (black bar) and indoors (grey bar) *Culicoides* relative composition in farms from Biscay (2010). On the left *C. obsoletus*/*C. scoticus* and on the right *C. pulicaris C. lupicaris*.



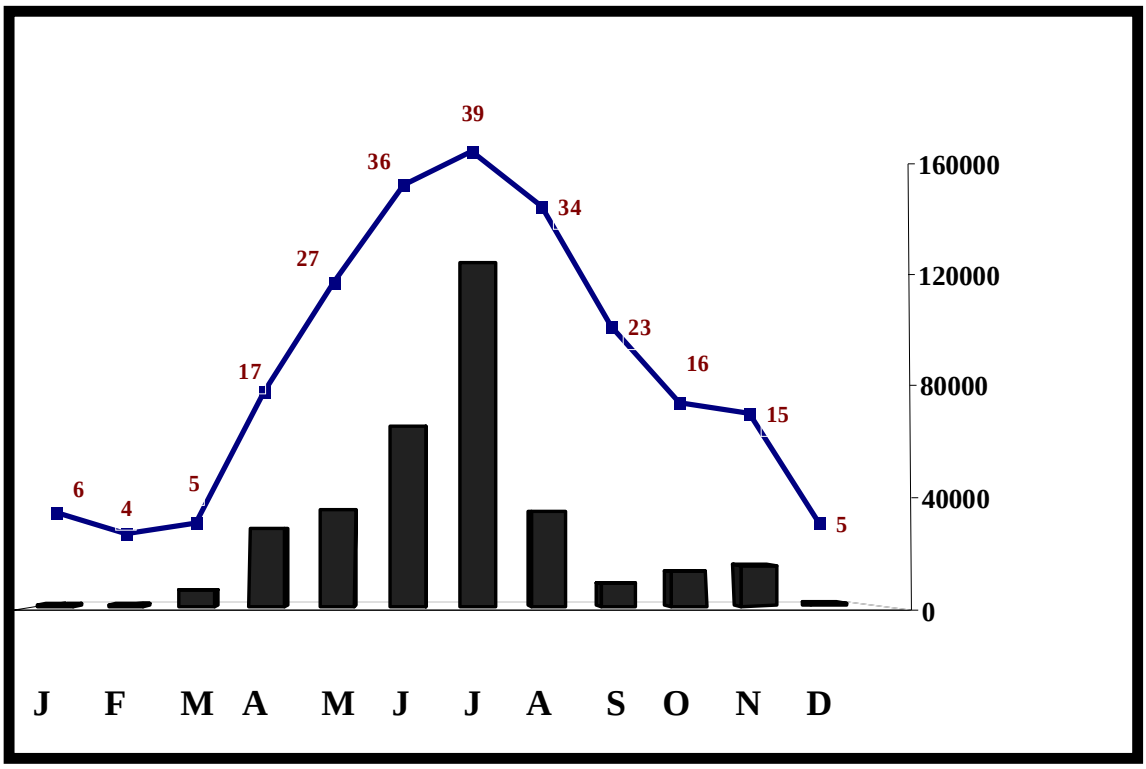
Appendix I (4). Sampling of the three sheep-farms (indoors + outdoors) in Gipuzkoa (year 2008) **(A)** Total *Culicoides* spp. specimens captured **(B)** Total number of *Culicoides* species captured **(C)** Total percentage species composition **(D)** Total *Culicoides* specimens distributed over the sampled farms.



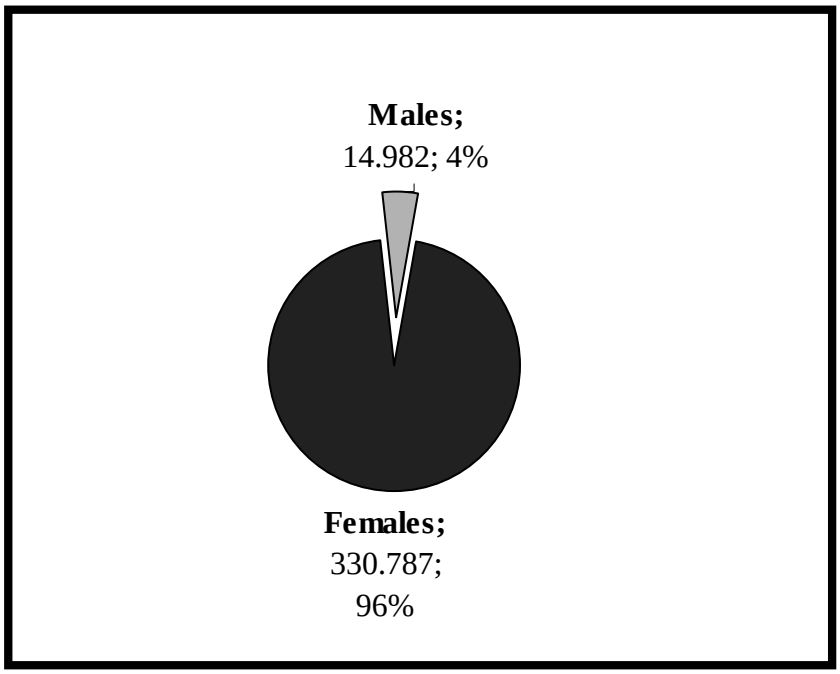
Appendix I (5). Sampling of the three sheep-farms (indoors + outdoors) in Alava (year 2009) **(A)** Total *Culicoides* spp. specimens captured **(B)** Total number of *Culicoides* species captured **(C)** Total percentage species composition **(D)** Total *Culicoides* specimens distributed over the sampled farms.



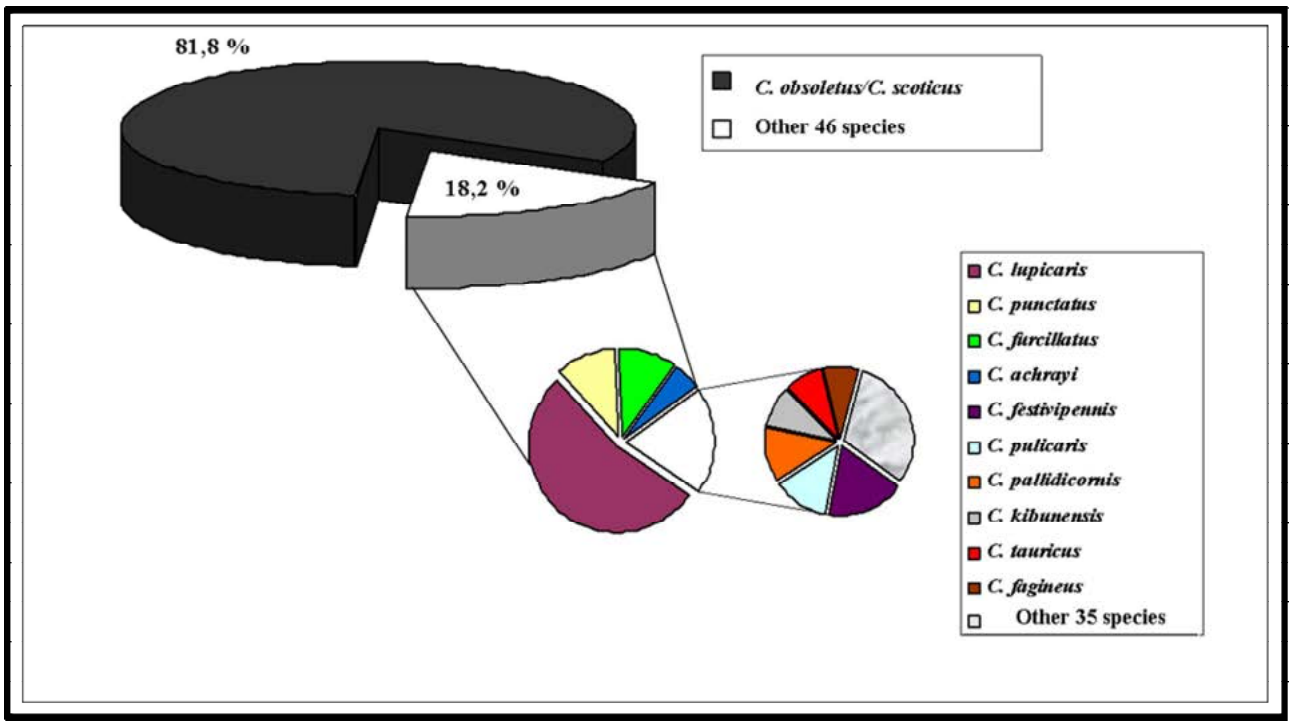
Appendix I (6). Sampling of the three sheep-farms (indoors + outdoors) in Biscay (year 2010) **(A)** Total *Culicoides* spp. specimens captured **(B)** Total number of *Culicoides* species captured. **(C)** Total percentage species composition **(D)** Total *Culicoides* specimens distributed over the sampled farms.



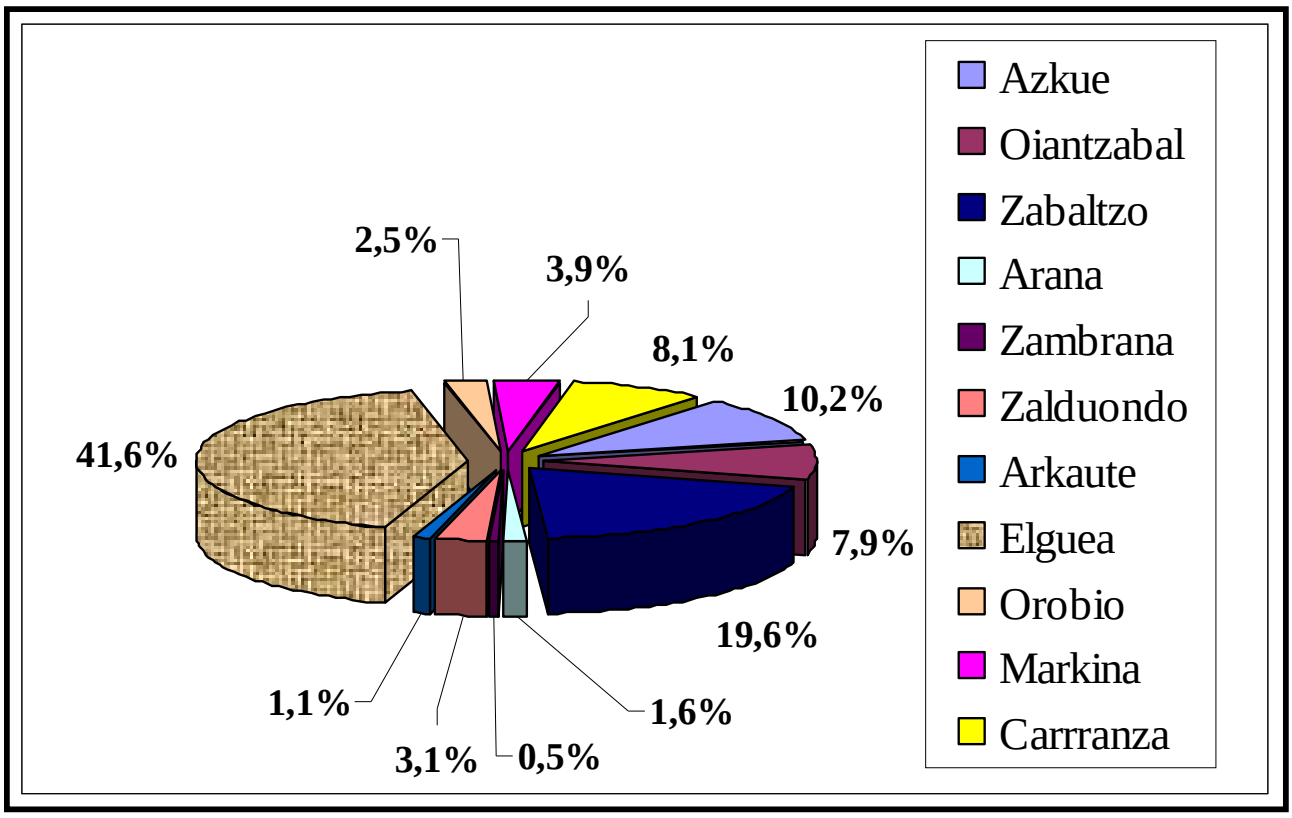
Appendix I (7). Histogram representing the total *Culicoides* midges abundance (black bars) in the eleven farms, outdoors + indoors pooled together, from the whole Basque Country farm settings. Blue line with red numbers represent the total number of species captured per month.



Appendix I (8). *Culicoides* sex ratio from the whole captured specimens at the Basque Country sheep-farms.



Appendix I (9). *Culicoides* species distribution from the whole farm setting sampling sites.



Appendix 1 (10). Percentage of *Culicoides* specimens distributed in the sampled farms.

2. NATURAL SETTINGS

i) WETLAND

Appendix I (11). Wetland in winter season (Salburua, Alava, Basque Country).

Table 1. Total catches of *Culicoides* species in the wetland of Salburua

WETLAND	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.obsoletus	0	0	0	0	0	0	0	0	0	0	1	0	1
C.scoticus	0	1	0	0	0	0	0	0	0	0	0	0	1
C.pulicaris	1	0	0	0	0	0	0	0	0	0	0	0	1
C.punctatus	1	0	0	0	2	0	1	1	0	0	2	0	7
C.alazanicus	0	0	2	0	0	1	3	0	1	3	3	0	13
C.clastrieri	0	0	0	0	0	0	1	0	0	0	0	0	1
C.duddingstoni	0	0	0	0	1	1	0	1	0	0	0	0	3
C.festivipennis	0	0	1	1	0	1	3	0	2	2	25	6	41
C.griseidorsum	2	0	2	0	2	0	1	0	0	0	0	0	7
C.kibunensis	0	0	0	0	2	0	4	0	0	0	0	0	6
C.pictipennis	0	0	1	0	0	0	0	0	0	0	0	0	1
C.poperinghensis	6	2	1	0	2	0	0	0	0	0	0	0	11
C.univitattus	1	0	0	0	0	0	0	0	0	0	0	0	1
TOTAL	**11**	**3**	**7**	**1**	**9**	**3**	**13**	**2**	**3**	**5**	**31**	**6**	**94**
	14		**8**		**12**		**15**		**8**		**37**		

ii) GRASSLAND

Appendix I (12). Grassland in winter season (Salburua, Alava, Basque Country).

Table 2. Total catches of *Culicoides* species in the grassland of Salburua.

GRASSLAND	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.dewulfi	0	0	2	0	0	2	0	0	0	0	0	0	4
C.obsoletus	0	0	1	0	2	0	0	0	0	0	1	0	4
C.scoticus	9	0	0	0	0	0	0	0	0	0	0	0	9
C.lupicaris	0	0	1	0	0	0	0	0	0	0	0	0	1
C.newsteadi	0	0	0	0	0	0	0	0	0	0	1	0	1
C.punctatus	1	0	2	0	0	1	2	1	6	2	2	8	25
C.alazanicus	0	0	8	0	13	0	45	1	29	1	45	5	147
C.brunnicans	0	0	1	0	0	0	0	0	0	0	0	0	1
C.duddingstoni	0	0	1	1	0	0	0	0	0	0	0	0	2
C.festivipennis	0	0	4	0	1	0	11	2	19	3	118	18	176
C.gejgelensis	0	0	0	0	1	0	1	0	0	0	0	0	2
C.griseidorsum	0	0	0	0	2	0	0	0	0	0	0	0	2
C.kibunensis	0	0	2	0	3	1	2	0	2	0	0	0	10
C.maritimus	0	0	0	0	0	0	0	1	0	0	0	0	1
C.pictipennis	1	0	2	1	0	0	0	0	0	0	0	0	4
C.poperinghensis	0	4	31	15	1	0	0	0	0	0	0	0	51
C.univitattus	0	0	5	0	0	0	0	0	0	0	0	0	5
TOTAL	**11**	**4**	**60**	**17**	**23**	**4**	**61**	**5**	**56**	**6**	**167**	**31**	**445**
	15		**77**		**27**		**66**		**62**		**198**		

iii) OAK GROVE

Appendix I (13). Oak grove (Cerio, Alava, Basque Country).

Table 3. Total catches of *Culicoides* species in the oak grove of Cerio.

OAK GROVE	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.obsoletus	0	0	1	0	0	0	1	2	0	1	5	0	10
C.scoticus	0	0	1	0	0	0	0	0	0	0	0	0	1
C.lupicaris	0	0	3	2	1	0	0	0	0	0	0	0	6
C.newsteadi	0	0	0	0	1	0	0	0	0	0	0	0	1
C.punctatus	0	0	45	1	9	0	1	0	0	0	0	0	56
C.alazanicus	0	0	0	3	14	6	8	4	10	3	12	17	77
C.brunnicans	0	0	1	8	0	0	0	0	0	0	0	0	9
C.duddingstoni	0	0	0	1	0	0	1	0	0	0	0	0	2
C.festivipennis	0	0	2	6	0	4	1	3	2	1	11	7	37
C.furcillatus	0	0	0	0	0	0	1	0	0	0	0	0	1
C.kibunensis	0	0	0	0	15	7	26	1	5	1	0	0	55
C.pictipennis	2	0	86	71	1	0	0	1	0	1	0	0	162
C.poperinghensis	0	0	2	1	0	0	0	0	0	0	0	0	3
C.pseudoheliophilus	4	4	2	0	0	0	0	0	0	0	0	0	10
C.univitattus	2	4	97	84	6	0	0	0	0	0	0	0	193
C.vexans	0	0	0	1	0	0	0	0	0	0	0	0	1
C.achrayi	0	0	2	1	1	0	0	0	1	0	0	0	5
TOTAL	**8**	**8**	**242**	**179**	**48**	**17**	**39**	**11**	**18**	**7**	**28**	**24**	**629**
	16		**421**		**65**		**50**		**25**		**52**		

iv) MEDITERRANEAN SHRUBLAND

Appendix I (14). Mediterranean shrubland (Etura, Alava, Basque Country).

Table 4. Total catches of *Culicoides* species in the Mediterranean shrubland of Etura.

MEDITERRANEAN SHRUBLAND	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.dewulfi	0	0	0	1	0	0	0	0	0	0	0	0	1
C.obsoletus	0	0	1	3	3	4	2	1	3	0	7	0	24
C.scoticus	0	0	2	0	0	1	0	0	0	0	0	0	3
C.lupicaris	0	0	2	0	0	1	0	0	0	0	0	0	3
C.punctatus	0	0	0	0	1	0	0	0	0	0	0	0	1
C.alazanicus	0	0	1	0	0	0	0	0	1	0	1	0	3
C.brunnicans	0	0	50	6	19	9	0	0	0	0	0	0	84
C.duddingstoni	0	0	1	0	0	0	0	0	0	0	0	0	1
C.kibunensis	0	0	3	1	9	5	2	0	0	0	0	0	20
C.poperinghensis	0	0	0	2	0	0	0	0	0	0	0	0	2
C.pseudoheliophilus	0	0	0	1	0	0	0	0	0	0	0	0	1
C truncorum	0	0	0	1	0	0	0	0	0	0	0	0	1
C.univitattus	0	0	3	0	0	0	0	0	0	0	0	0	3
C.vexans	0	0	6	0	2	1	0	0	0	0	0	0	9
TOTAL	**0**	**0**	**69**	**16**	**34**	**21**	**4**	**1**	**4**	**0**	**8**	**0**	**156**
	0		**85**		**55**		**5**		**4**		**8**		

v) POND

Appendix I (15). Pond (Barrundia, Alava, Basque Country).

Table 5. Total catches of *Culicoides* species in the pond of Barrundia.

POND	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.dewulfi	0	0	0	0	0	0	0	0	0	0	0	1	1
C.obsoletus	1	1	2	1	3	2	2	0	1	1	8	0	22
C.scoticus	1	0	1	2	0	1	0	0	0	0	1	2	8
C.lupicaris	0	0	1	2	3	1	2	0	2	0	6	0	17
C.newsteadi	0	0	1	0	0	0	0	0	0	0	0	0	1
C.pulicaris	0	0	0	0	0	2	0	0	0	0	0	0	2
C.punctatus	0	0	45	4	12	2	10	0	0	0	2	2	77
C.brunnicans	0	0	11	16	2	0	0	0	0	0	0	0	29
C.festivipennis	0	0	24	1	9	8	7	1	8	1	59	13	131
C.kibunensis	0	0	0	0	2	1	8	0	0	0	0	0	11
C.pictipennis	0	0	13	2	1	0	0	0	0	0	0	0	16
C.poperinghensis	0	0	6	11	1	0	0	0	0	0	0	0	18
C.pseudoheliophilus	0	0	6	2	1	1	0	0	0	0	0	0	10
C.shaklawensis	0	0	0	0	3	1	0	0	0	0	0	0	4
C.simulator	0	0	2	1	3	0	0	0	0	0	0	0	6
C.univitattus	1	0	2	0	1	0	0	0	0	0	0	0	4
C.vexans	0	0	1	0	1	0	0	0	0	0	0	0	2
C.achrayi	0	0	26	22	27	9	1	0	0	0	0	0	85
C.fascipennis	0	0	0	0	1	0	0	0	0	0	2	0	3
C.pallidicornis	0	0	0	0	1	0	0	0	0	0	0	0	1
C.picturatus	0	0	11	0	5	9	0	0	0	0	0	0	25
TOTAL	**3**	**1**	**152**	**64**	**76**	**37**	**30**	**1**	**11**	**2**	**78**	**18**	**473**
	4		**216**		**113**		**31**		**13**		**96**		

vi) PINE FOREST

Appendix I (16). Pine forest (Barrundia, Alava, Basque Country).

Table 6. Total catches of *Culicoides* species in the pine forest of Barrundia.

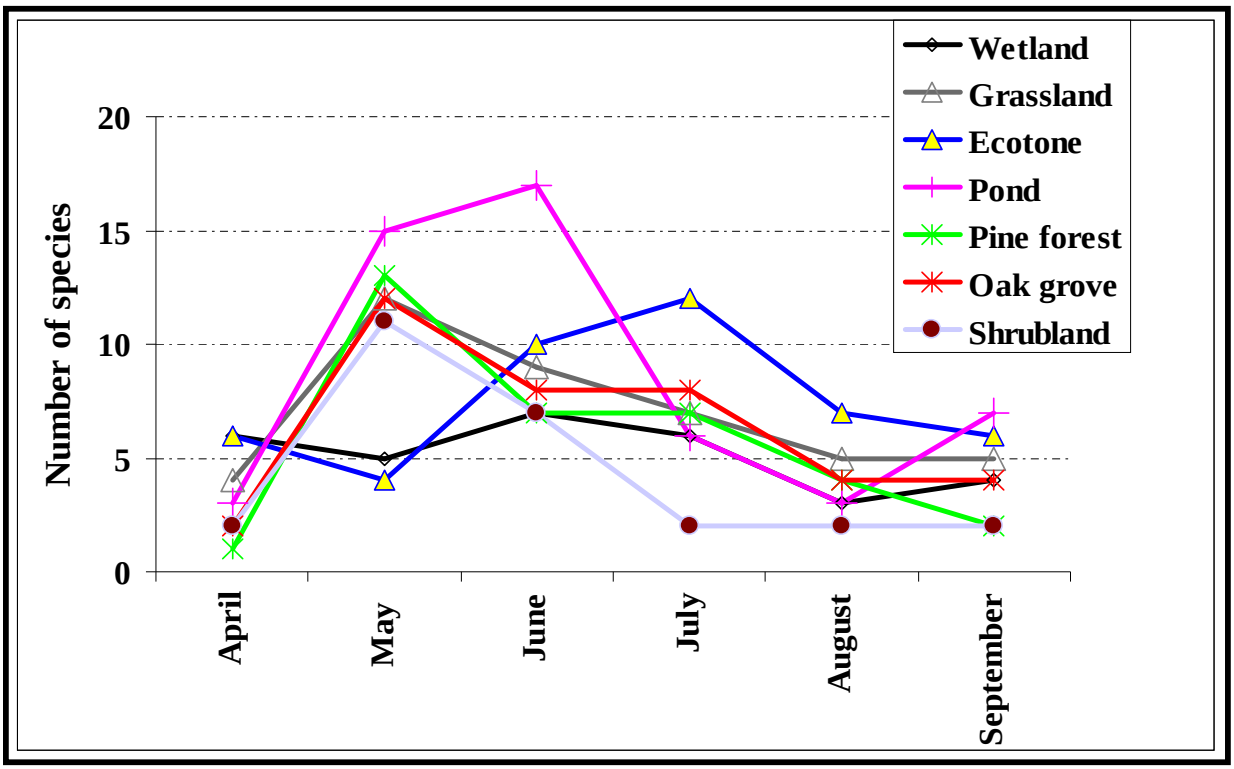
PINE FOREST	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.obsoletus	0	0	11	0	2	0	23	14	1	1	4	0	56
C.scoticus	0	1	0	0	0	0	4	3	0	0	0	0	8
C.lupicaris	0	0	6	0	1	0	1	0	1	0	2	0	11
C.punctatus	0	0	40	0	1	0	1	0	0	0	0	0	41
C.brunnicans	0	0	175	2	2	0	0	0	0	0	0	0	179
C.festivipennis	0	0	0	1	0	0	0	0	0	0	0	0	1
C.furcillatus	0	0	0	0	1	0	0	0	0	0	0	0	1
C.heliophilus	0	0	1	0	0	0	0	0	0	0	0	0	1
C.longipennis	0	0	0	0	0	0	0	0	1	0	0	0	0
C.kibunensis	0	0	0	0	0	0	5	0	2	0	0	0	7
C.pictipennis	0	0	7	0	0	0	0	0	0	0	0	0	7
C.poperinghensis	0	0	4	0	0	0	0	0	0	0	0	0	4
C.pseudoheliophilus	0	2	0	0	0	0	0	0	0	0	0	0	2
C.simulator	0	0	3	0	0	0	0	0	0	0	0	0	3
C.univitattus	0	0	1	0	0	0	0	0	0	0	0	0	1
C.achrayi	0	0	42	8	12	3	28	0	0	0	0	0	93
C.fascipennis	0	0	4	0	0	0	1	0	0	0	0	0	5
C.picturatus	0	0	4	0	0	0	0	0	0	0	0	0	4
TOTAL	**0**	**3**	**298**	**11**	**19**	**3**	**63**	**17**	**5**	**1**	**6**	**0**	**426**
	3		**309**		**22**		**80**		**6**		**6**		

vii) ECOTONE

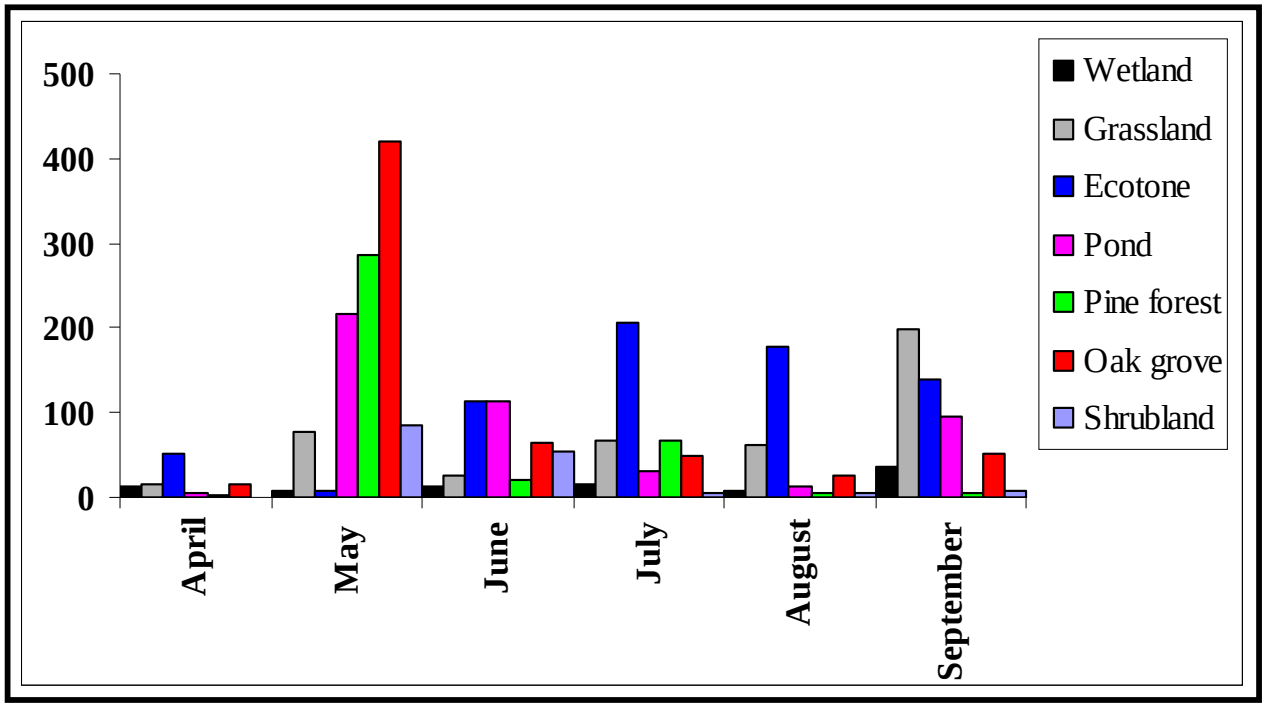
Appendix I (17). Ecotone (Arkaute, Alava, Basque Country).

Table 7. Total catches of *Culicoides* species in the ecotone of Arkaute.

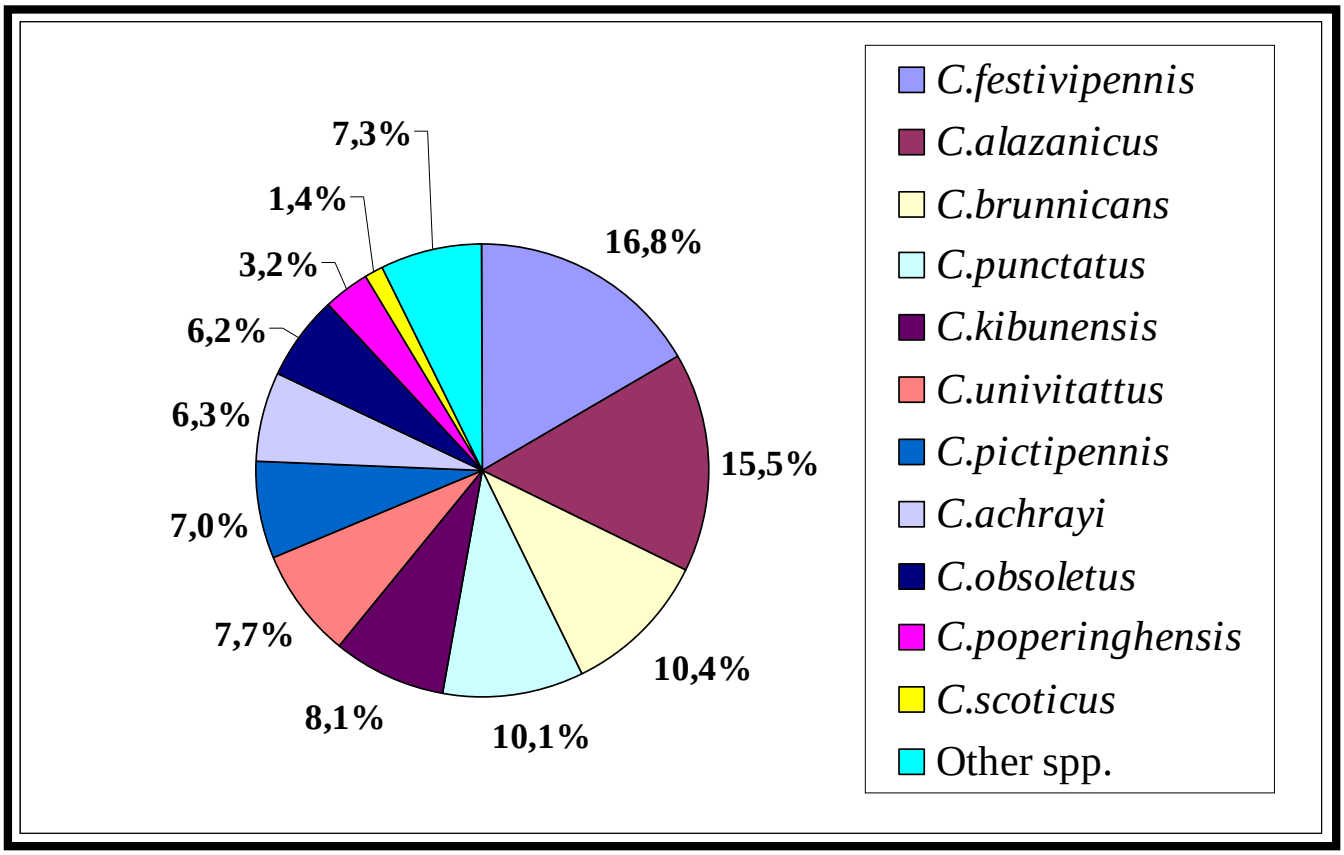
ECOTONE	April		May		June		July		August		September		Total
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	
C.dewulfi	0	0	0	0	0	0	0	0	1	0	0	0	1
C.obsoletus	1	0	2	2	3	3	8	10	12	6	10	6	63
C.scoticus	5	2	0	0	0	0	0	0	1	0	2	0	10
C.circumscriptus	0	0	0	0	1	0	0	0	0	0	0	0	1
C.fagineus	0	0	0	0	0	1	0	0	0	0	0	0	1
C.lupicaris	0	0	0	0	0	0	0	1	0	0	0	0	1
C.newsteadi	0	0	0	0	0	0	1	0	0	0	0	0	1
C.punctatus	0	0	1	0	5	4	60	8	4	1	4	1	88
C.alazanicus	0	0	0	0	17	2	42	6	82	0	63	0	212
C.brunnicans	0	0	0	0	0	0	1	0	0	0	0	0	1
C.duddingstoni	0	0	1	0	9	10	1	2	0	0	0	0	23
C.festivipennis	0	0	0	0	4	1	7	6	45	3	35	3	104
C.furcillatus	0	0	0	0	0	0	1	0	0	0	0	0	1
C.gejgelensis	0	0	0	0	0	0	1	0	0	0	0	0	1
C.griseidorsum	0	0	0	0	10	0	0	0	2	0	0	0	12
C.kibunensis	0	0	0	0	39	4	42	7	21	0	13	0	126
C.maritimus	0	0	0	0	0	0	1	1	0	0	0	0	2
C.pictipennis	8	6	1	0	0	0	0	0	0	0	0	0	15
C.poperinghensis	3	0	0	0	0	0	0	0	0	0	0	0	3
C.simulator	0	0	0	0	1	0	0	0	0	0	0	0	1
C.univitattus	14	9	0	0	0	0	0	0	0	0	0	0	23
C.vexans	2	2	0	0	0	0	0	0	0	0	0	0	4
TOTAL	**33**	**19**	**5**	**2**	**89**	**25**	**165**	**41**	**168**	**10**	**127**	**10**	**694**
	52		**7**		**114**		**206**		**178**		**137**		



Appendix I (18). Number of *Culicoides* species captured per month in the natural settings (from April to September 2011).



Appendix I (19). Each bar represents the total the total *Culicoides* specimens captured in the natural habitats per month (two non-stop night collections per week).



Appendix I (20). Distribution of the most common *Culicoides* species in the whole natural habitats.

APPENDIX II (CHAPTER 3)

1. EXTRA PICTURES ABOUT THIS CHAPTER

Appendix II (1). Home-made emergence traps.

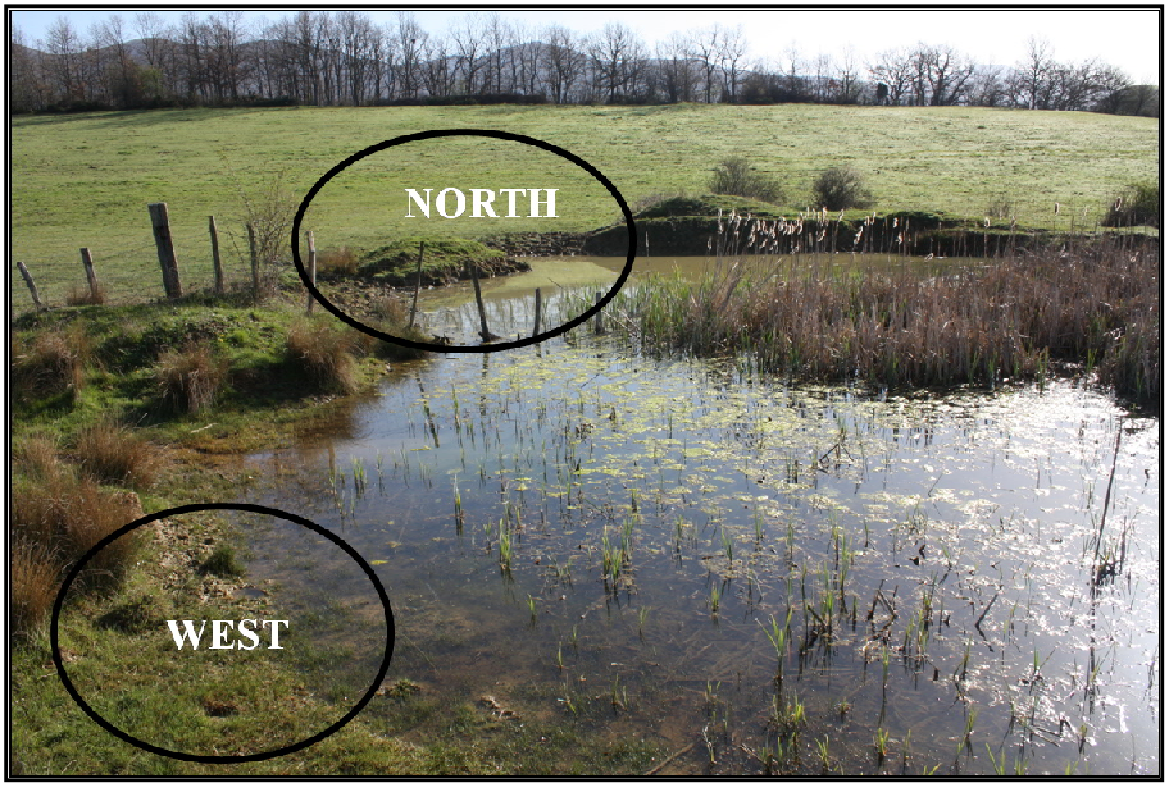
Appendix II (2). Detail of the jar with collected insects.

Appendix II (3). Iron-corer.

Appendix II (4). Panoramic of the farm (Elguea, Álava). Star indicates CDC-UV light trap position.

Appendix II (5). Panoramic view of the poplar grove (Elguea, Álava). The river is located behind trees.

Appendix II. Some *Culicoides* breeding sites **(6)** Heap of manure; black arrow pointing to old-composted manure and red arrow pointing to fresh manure **(7)** Manure and organic matter on farm building **(8)** Poplar grove soil **(9)** Fallen leaves with *Lathraea clandestina* **(10)** Trench; black arrows pointing runoff areas.



Appendix II (11). Panoramic view of the pond showing two regions: north pond and west pond, the other three pond sampling sites are located on the right of the photograph (Elguea, Álava).

Appendix II (12-13). Samplings with no *Culicoides* emergences; tree holes and pasture, respectively (Elguea, Álava).

APPENDIX III (CHAPTER 4)

1. LABORATORY ATTRACTION TRIALS

Table 1. Compounds and/or mixtures evaluated against *Culicoides obsoletus* in laboratory assays (2-choice olfactometer).

N	Commercial name	Category	Composition/Formula	Purity
1	**(DL)-Lactic acid**	Acid	$CH_3CH(OH)COOH$	90%
2	**L (+)-Lactic acid**	Acid	$CH_3CH(OH)COOH$	92%
3	**Acetone**	Ketone	CH_3COCH_3	≥99.5%
4	**1-Octen-3-ol**	Alcohol	$CH_3(CH2)_4CH(OH)CH=CH_2$ (sum of enantiomers)	≥98%
5	**Dichloromethane**	Organic compound	CH_2Cl_2	99%
6	**Ammonia**	Nitrogen compound	NH_3	-
7	**Sesame seeds**	Plant derived	*Sesasum indicum*	90%
8	**p-cresol**	Phenol	$CH_3C_6H_4OH$	≥98%
Mix 1	**Mixture 1:1 (L-lactic + dichloromethane)**	Mixture	Mixture	-

Table 2. Percentage of attraction obtained with eight single compounds and one mixture, at 1 µg/µl, 0.1µg/µl, 0.01 µg/µl, for *Culicoides obsoletus* as determined with a Y-tube olfactometer.

Compound	1 µg/µl			0.1/µl			0.01 µg/ µl			
	C	**T**	**P-value**	**C**	**T**	**P-value**	**C**	**T**	**P-value**	**+**
(DL)-Lactic acid	48.0	52.0	0.124	57.3	46.7	0.225	58.3	42.7	0.248	90.4
L (+)-Lactic acid	69.2	31.8	0.005	52.9	47.1	0.731	41.1	58.9	0.303	84.0
Acetone	52.5	47.5	0.752	54.1	45.9	0.564	38.2	68.8	0.017	98.2
1-octen-3-ol	67.5	32.5	0.026	52.7	47.7	0.738	52.6	47.4	0.263	93.6
Dichloromethane	45.5	55.5	0.467	39.4	59.6	0.085	41.6	58.4	0.157	97.5
Ammonia	45.0	55.0	0.371	55.2	44.8	0.202	52.5	47.5	0.751	95.7
Sesame seeds	58.0	42.0	0.204	50.0	50.0	1.000	46.6	50.4	0.605	96.3
Mixture 1	45.2	44.8	1.000	52.5	48.5	0.875	49.3	51.7	0.874	91.6

Percentage response (+): uplight orientation plus choice. P value was obtained with Chi-square test (α= 0.05). N=100-120 specimens per treatment.

Table 3. Specific odours evaluated for *Culicoides obsoletus* in laboratory assays (2-choice olfactometer).

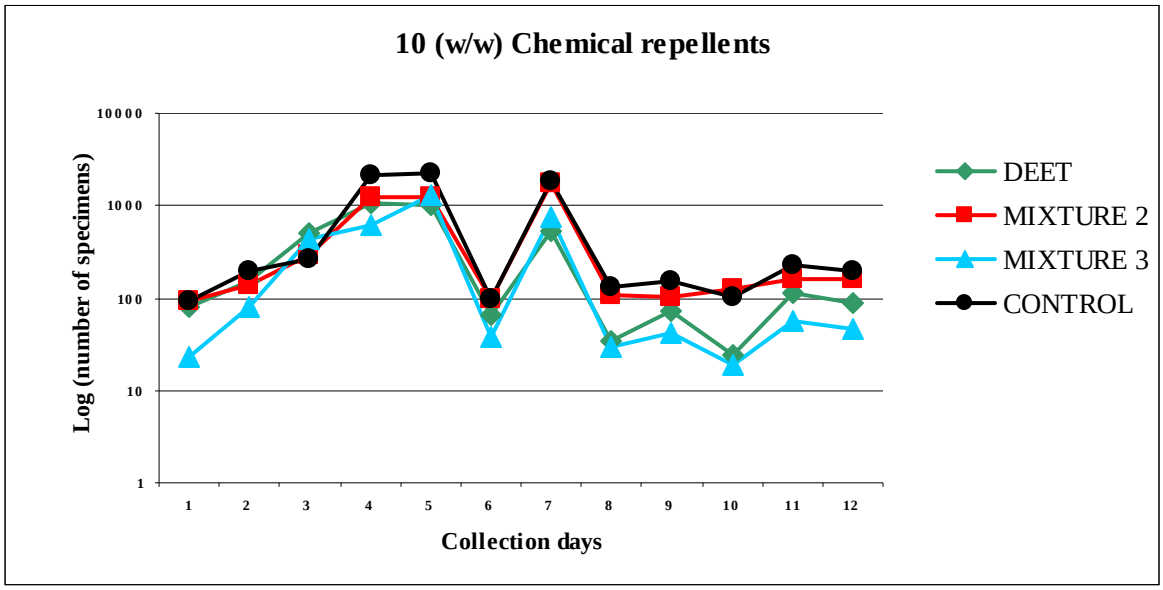
	Odours	Quantity	Description	Sex
1	**Sheep hairs**	3 µl	Washes in hexane	♀
2	**Sheep hairs**	3 g	Fresh	♀
3	**400 specimens of field *C. obsoletus* females**	3 µl	Freshy squashed diluted in 1 ml hexane	♀;♂
4	**Specimens of field *C. obsoletus* females**	100	Alive adults	♀;♂
5	**Farm odour**	3 µl	Extract in hexane from cotton placed under fan traps	♀
6	**Fresh manure**	3 g	Yellowish (only few days of fermentation)	♀
7	**Composted- manure**	3 g	Dark coloured (one year old)	♀

Table 4. Percentage of attraction obtained with specific odours for *Culicoides obsoletus* in laboratory assays (2-choice olfactometer)

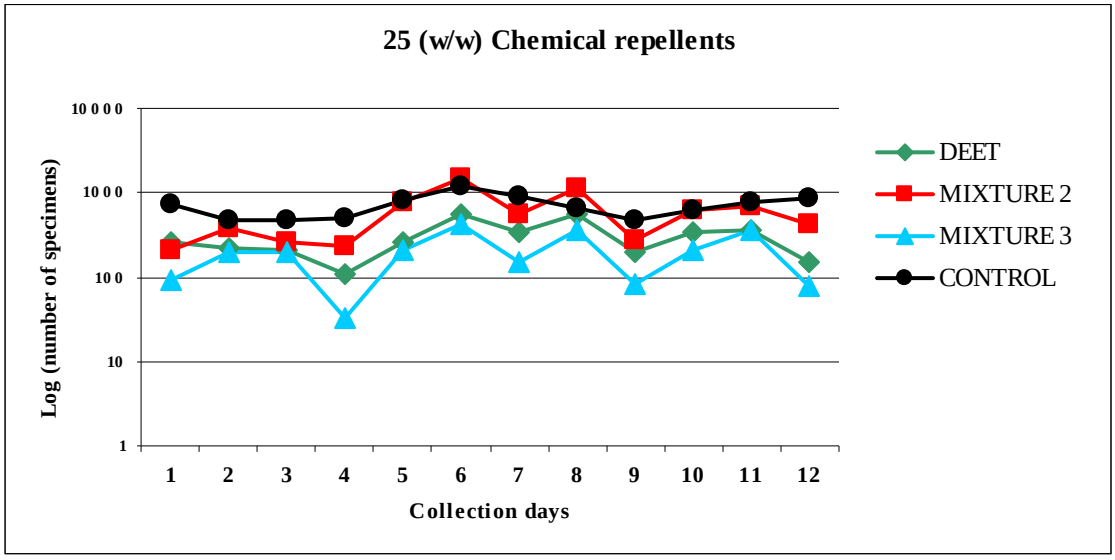
Odour	C	T	P-value	Sex tested	+
Sheep hairs (hexane)	62.7	37.3	0.017	♀	84.0
Sheep hairs (fresh)	62.6	37.4	0.020	♀	86.2
***C. obsoletus* females (hexane)**	48.7	51.3	0.820	♀	71.6
***C. obsoletus* females (alive)**	45.4	54.6	0.669	♂	83.3
Farm odour (hexane)	48.8	52.0	0.825	♀	95.3
Fresh manure	48.8	51.2	0.805	♀	86.8
Composted-manure	45.0	55.0	0.527	♀	80.0

Percentage response (+): uplight orientation plus choice. P value was obtained with Chi-square test (α= 0.05). N=100-120 specimens per treatment.

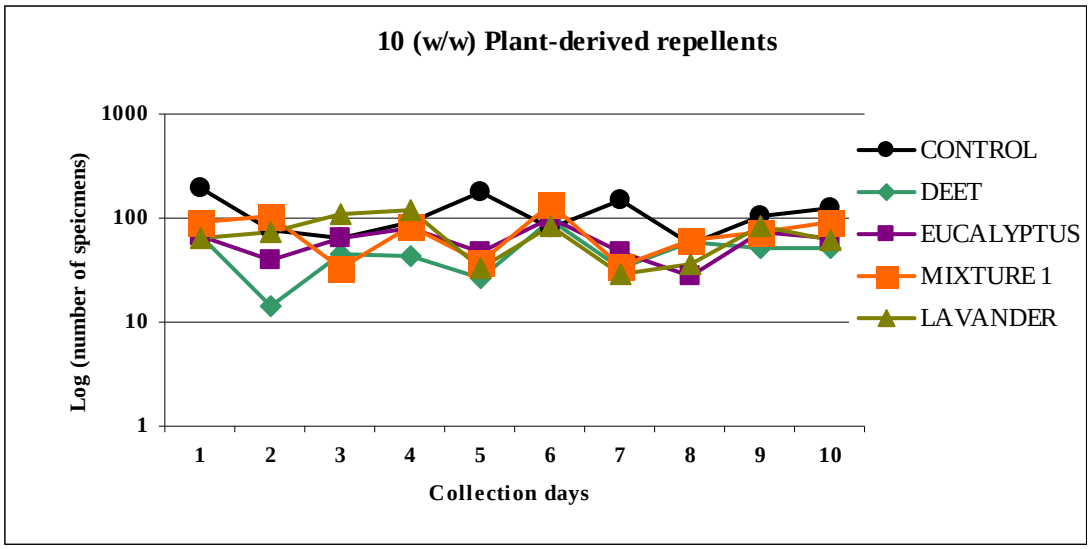
2. FIELD REPELLENCE TRIALS



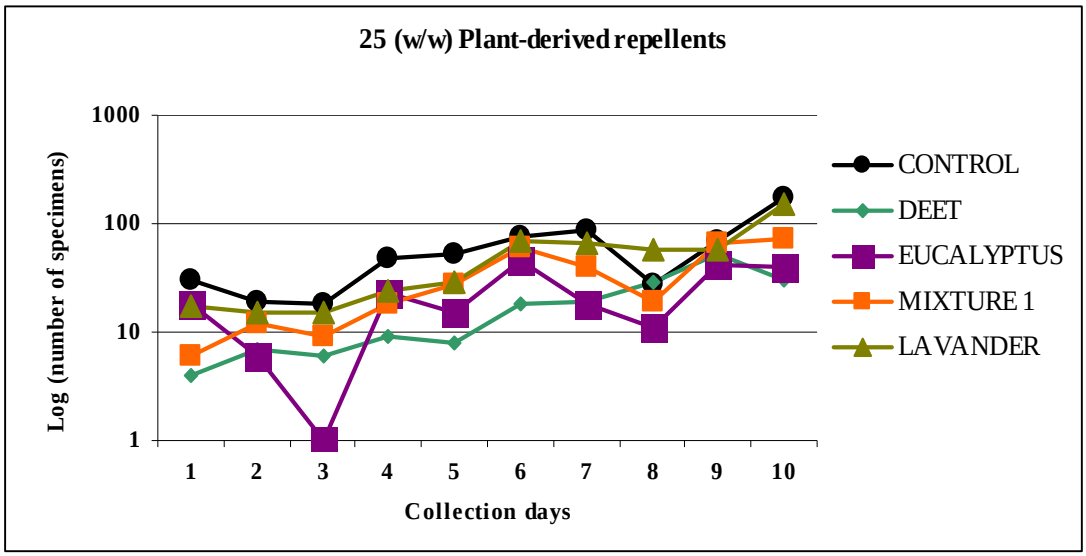
Appendix III (1). Number of *Culicoides* spp. over 12 collection days (5 night hours per day) with light traps fitted with polyester mesh treated at 10% of repellent concentration of DEET, mixture 2 (geranyl acetone plus 6-methyl-5-hepten-one), mixture 3 (octanoic plus nonanoic plus decanoic) and a control.



Appendix III (2). Number of *Culicoides* spp. over 12 collection days (5 night hours per day) with light traps fitted with polyester mesh treated at 25% of repellent concentration of DEET, mixture 2 (geranyl acetone plus 6-methyl-5-hepten-one), mixture 3 (octanoic plus nonanoic plus decanoic) and a control.



Appendix III (3). Number of *Culicoides* spp. over 10 collection days (5 night hours per day) with light traps fitted with polyester mesh treated at 10% of repellent concentration of DEET, eucalyptus, mixture 1 (jasmine, lavander, rosemary), lavander and a control.



Appendix III (4). Number of *Culicoides* spp. over 10 collection days (5 night hours per day) with light traps fitted with polyester mesh treated at 25% of repellent concentration of DEET, eucalyptus, mixture 1 (jasmine, lavander, rosemary), lavander and a control.

APPENDIX IV (CHAPTER 5)

1. ATTEMPTS TO DETECT THE PHEROMONE *N*-HEPTADECANE OF *CULICOIDES NUBECULOSUS*

- INTRODUCTION

Although not all midges have sex pheromones, as recognition of females by males in may instances occurs by auditory recognition of the female by the male using her wing beat frequently as the females fly into male leks. To date, the role of pheromones have not been discovered in many *Culicoides* midges except in the case of *C. nubeculosus, C. impunctatus* and *C. melleus* species (see **Chapter 5**). In order to detect the pheromone of *C. nubeculosus* we performed some laboratory assays.

- OBJECTIVE

The main objective was to detect, optimize and quantified the pheromone emitted by *Culicoides nubeculosus* females of different ages, using volatile collection techniques.

- MATERIAL AND METHODS

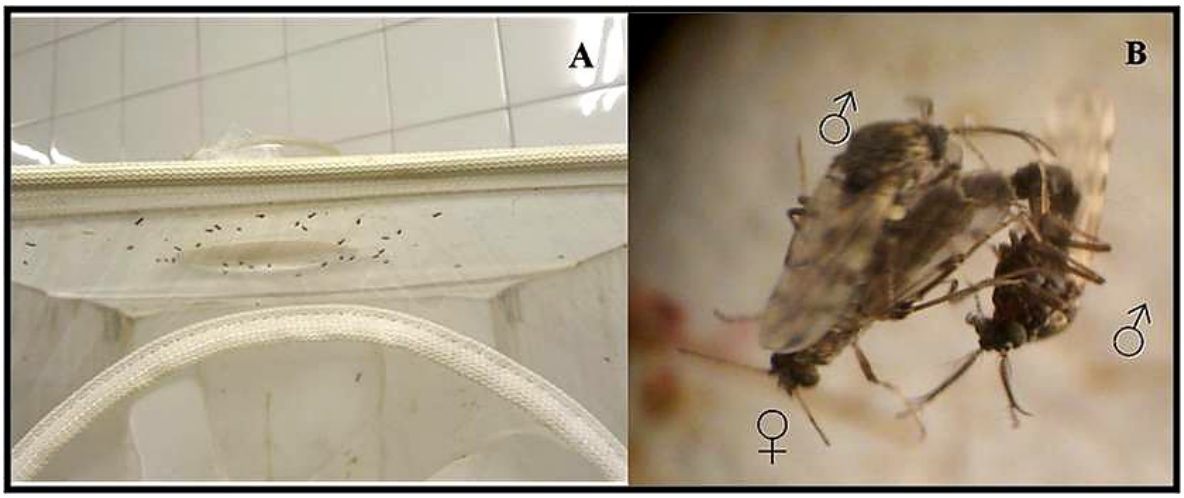
A colony of *C. nubeculosus* was kindly provided by the Institute of Animal Health (IAH), Pirbright, U.K. A pot of males and females respectively of this species composed of 200-300 specimens were placed on cubic mosquito nets until their emergence. Three times per day, emerged specimens were separated according to their age. Midges were maintained at 23 ± 2º C, 80 ± 5% relative humidity and invert photoperiod (12/12 h). 10% of sucrose solution was added in cotton balls (Appendix IV: 1A). Three different trials at the same time repeated in three different days (replicates) corresponded to 24 and 48 hours of alive *C. nubeculosus* were carried out.

The experiment can be summarised as follows: A 500 ml/min air-flow was passed over two Erlenmeyer flasks (the first one containing water or blood and the second one empty). Two meshes were placed on between inlet and outlet glass chambers containing *Culicoides* specimens. Another piece of glass was joined to the main chamber which connects SPME-fiber and Porapak cartridges (see details of them in the previous section) at the same time (Appendix IV: 2). Heparinised blood of rabbit was used as odour to stimulate the sex

pheromone production (Appendix IV: 2C). The experiment in all trials latest six hours and chambers were covered with tinfoil for partly darkness conditions. The experiment consisted on a control (without insects, first Erlenmeyer flask contains water), a group of midges (Erlenmeyer flask containing water) and the third group of midges (Erlenmeyer flask containing blood). In the third group, prior to start the experiment, blood was heated to 37º C in a boiling water bath, then 15 ml of blood were added to Erlenmeyer flask and 30 min of a flow of air (with blood odours) passing over specimens sample.

Following replicates were performed:

i) First replicate (24 h old) consisted on 60 females of *C. nubeculosus* in each sample except control.
ii) Second replicate (48 h old) consisted on 60 females of *C. nubeculosus* in each sample except control.
iii) Third replicate (48 h old) consisted on mixed (30 males and 30 females) of *C. nubeculosus* in each sample except control.



Appendix IV (1). *Culicoides nubeculosus* emerged adults **(A)** Cotton balls impregnated with sucrose solution **(B)** Attempts of mating of two males with a single female.

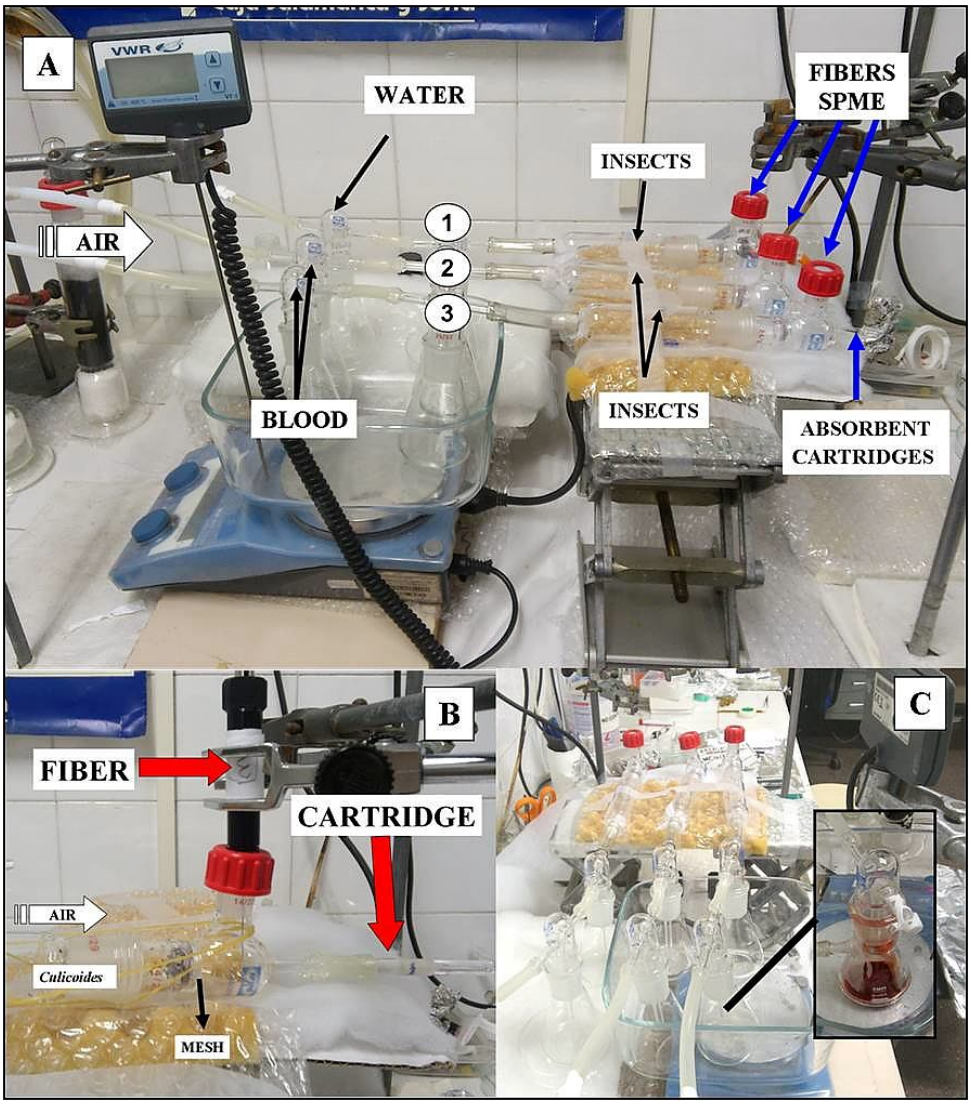
- RESULTS

After analyzing all the chromatograms no evidence of increasing amounts *n*-heptadecane were detected. No other compounds of biological interest were found with none of the both methodologies used. We observed attempts of mating between males-males, male-female and various male with a single female (Appendix IV: 1B).

- CONCLUSIONS

In light of the disappointing results various plausible reasons can be summarised:

- The pheromone production is negligible and these techniques are useless to detect such emission. The colleagues of IACR-Rothamsted (UK) used an exclusive technology equipment (M, Birkett, personal communication).
- The number of *Culicoides* is scanty and various hundreds of specimens are necessary to detected changes in the pheromone production (J, Mordue, personal communication).
- A mistake in the choice of the correct physiological status "age" is excluded, because as has been reported, suitable age is between 24-48 post-emergence.
- It is also unlikely that the choice of rabbit blood instead of sheep blood was responsible of the unsuccessfull result, because in absence of blood odours the pheromone production is also produced according to reported works.



Appendix IV (2). Experiment designed to capture the pheromone of *Culicoides nubeculosus* **(A)** Overview **(B)** Detail of absorbent materials **(C)** Detail of the flask containing blood of rabbit.

2. PRELIMINARY ATTEMPTS TO FOUND THE POSSIBLE EXISTENCE OF VOLATILE COMPOUNDS EMITTED BY *CULICOIDES OBSOLETUS*

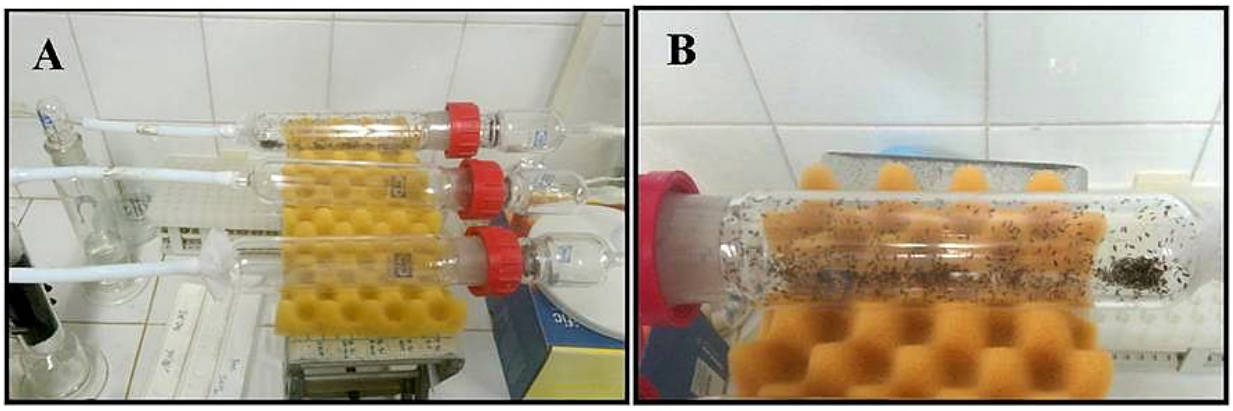
- OBJECTIVE

Elucidate if any volatile compounds are emitted by females of *Culicoides obsoletus* using techniques of absorbance.

- MATERIAL AND METHODS

A) Dynamic headspace volatile collection

Three glass chambers (two controls and a sample with specimens), 10 cm length and 2.5 cm i.d. with a dry/moisture charcoal-filtered air flow at a rate (350 ml/min) was operating during 24 h (12:12 h light-dark photoperiod; 22 ± 5º C; RH: 80%) at laboratory conditions. A mesh was placed on each side of the chambers to avoid that *Culicoides* escape. At the outlet chambers, Porapak-Q tubes (150/175 mg, 50/80 mesh; Supelco, Bellefonte, PA, USA) were used for the volatile collections (Appendix IV: 3). A total average of 250 wild caught females of *Culicoides obsoletus* s.l. were used in each replicate (mainly nulliparous and parous gonotrophic stages).



Appendix IV (3). Volatile collections by dynamic headspace extraction in glass tubes **(A)** General overview **(B)** Detail of *C. obsoletus* specimens inside the chamber.

After each replicat, the devices were cleaned with neutral soap, followed by baths in acetone and pure hexane and then were left to dryness the whole night. Three pseudoreplicates were performed:

i) Dry charcoal-filtered air passed over females placed on a chamber and the other ones as blanks. After 24 h, 90% of the specimens were found died.

ii) A glass saturated water-column was placed before the inlet to produce humid air. After 24 h, 10% of the specimens survived. A little layer of water remained on the chamber.

iii) A glass saturated water-column followed by another column as intermediate was placed before the inlet, to produce humidified air but not water-saturated. After 24 h, 80% of the specimens survived. Only few drops of water were observed inside the devices.

Porapak tubes were immediately rinsed with 2 ml of pure *n*-hexane (SupraSolv, Merck, Germany) to elute the volatiles. Glass samples (samples and controls) were stored at -20ºC prior to chemical analysis. A total of 500 µl of extract were concentrated under a gentle nitrogen stream to 2 µl and that was analysed by GC-MS (gas chromatography-mass spectrometry) on a Thermo Finnigan Trace 2000GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain). The column consisted of a HP-5MS (30 m × 0.25 mm i.d. × 0.25 µm; Agilent Technologies, Madrid, Spain) temperature programmed from 60° C (held for 1 min.) to 185° C at 5° C.min-1, then 5° C.min-1 to 200 º C, 5° C.min-1 to 270 º C (held for 10 min) and last ramp to 300 º C at 5° C min-1. Helium (1 ml.min-1) was the carrier gas. The MS was used in the electron impact (EI) mode at 70 eV, with a source temperatureof 200° C. The mass range scanned was 40–500, at 1.0 scan.sec-1, after a solvent delay of 4.0 min.

B) SPME (Solid-phase microextraction) assays

The volatile emanations of *Culicoides obsoletus* with this technique were also sampled. An average of 200 females of *Culicoides obsoletus* s.l. (mainly parous and nulliparous to a lesser extent) and two controls (without insects) were introduced into three clear glasses, wide-mouth septa jar (40 ml volume, 28 x 81 cm). SPME fibers 100µm, polydimethylsiloxane (Supelco Inc, Bellefonte, USA) were used at 22º C temperature during 20 h (10:10 h light-night photoperiod, RH: 80%). Prior to perform the sampling, SPME fibers were conditioned during 1 h at 250 degrees. Two assays were performed:

i) With glasses placed on vertical position, in order to avoid midges make contact with the coated fiber. After 20 h, all *Culicoides* specimens were found dead inside the glass bottles (Appendix IV, 4A).

ii) With glasses on horizontal position. In this way, insects were allowed to move more freely inside the glass and favoured the direct contact with the coated fiber. After 20 h, most *Culicoides* specimens remained alive (Appendix IV, 4B).

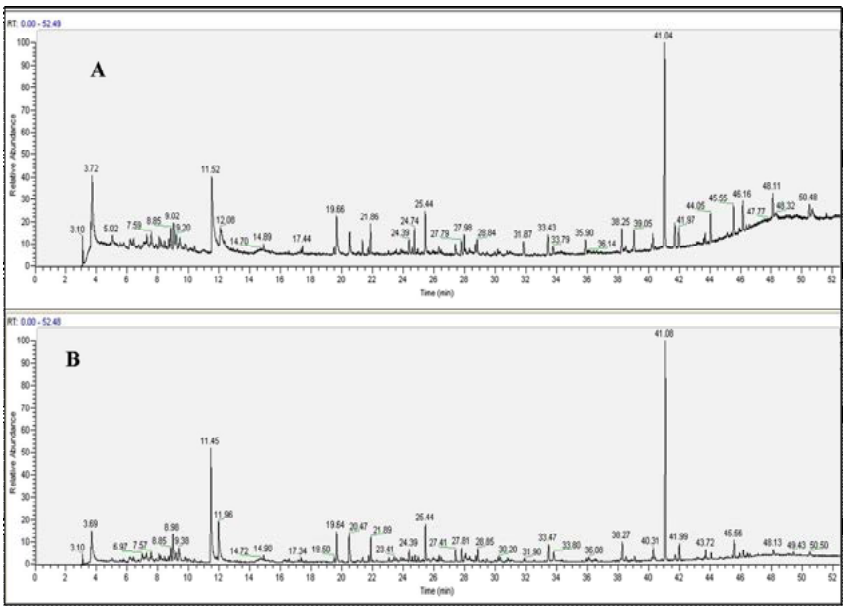
Appendix IV (4). SPME extraction analysis of *Culicoides obsoletus* **(A)** Vertical position **(B)** Horizontal position.

The fiber was thermally desorbed in the heated GC injection port for 1 min at 60º C and then ramped at 5º C min^{-1} to 270º C for 40 min. Injector and transfer line temperatures were 250 º C.

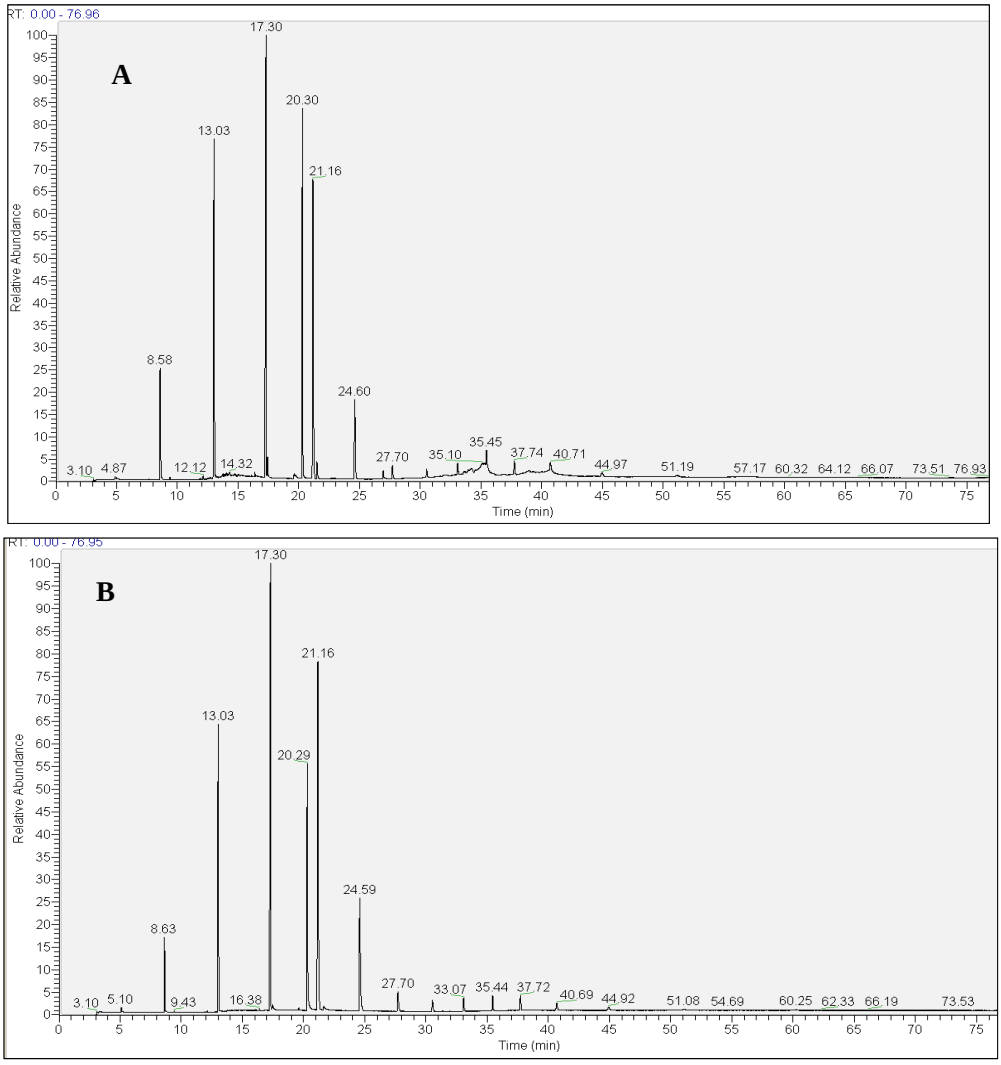
Both procedures (Dynamic headspace volatile collection and SPME) were analysed in the Department of Biological Chemistry and Molecular IQAC (CSIC) of Barcelona (Spain).

- RESULTS

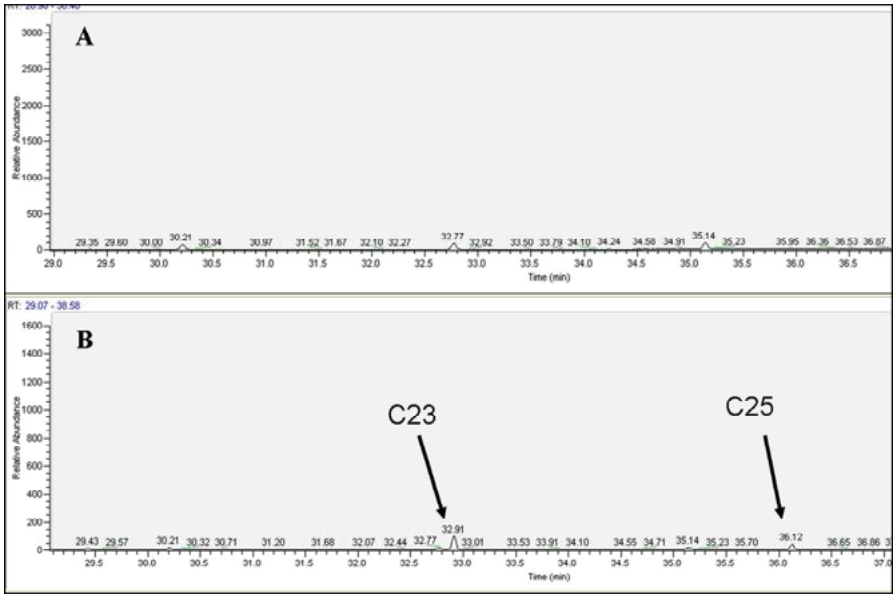
None compound of biological interest was found with any of the both mentioned methodologies (see example in Appendix IV: 5-6, identical chromatograms). In contrast, when *Culicoides* specimens took contact with the fiber, four small peaks were most likely correspond with *n*-alkanes present in their outer cuticular layer: C23, C25, C27 and C29 (Appendix IV: 7). We never observed attempts to copulate in laboratory conditions.



Appendix IV (5). GC-MS chromatograms from Porapak-cartridges **(A)** Control (without insects) **(B)** *Culicoides obsoletus* sample. Peaks represented in both pictures correspond with typical peaks derived from contamination of septum and/or stationary.



Appendix IV (6). GC-MS chromatograms from SPME-fiber **(A)** *Culicoides obsoletus* sample **(B)** Control (without insects). Peaks represented in both pictures correspond with typical peaks derived from contamination of septum and/or stationary.



Appendix IV (7). GC-MS chromatograms from SPME-fiber **(A)** Control (without insects) **(B)** *Culicoides obsoletus* sample. The peaks (only partly represented) correspond with detached linear hydrocarbons most likely from the cuticle of the legs.

- CONCLUSIONS

In light of the disappointing results various plausible reasons can be summarised:

- The techniques, devices and conditions here used are inadequate or not enough powerful to detect the volatile compounds.
- The artificial and unrealistic laboratory conditions are unfavourable for compounds emission.
- The mating behaviour of *C. obsoletus* is limited to visual stimulus or to the intervention of non-volatile compounds.
- The presence of other cues (visual, olfactory, markers) is essential for compounds production and emission. This hypothesis is reinforced with the fact that no mating has been observed in *C. obsoletus* at artificial conditions.