Universitat
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TESIS DOCTORAL

2019

Bionomía y modelos de abundancia estacional de las especies del género *Culicoides* (Diptera; Ceratopogonidae) en España, con especial interés en los vectores del virus de la Lengua Azul

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Doctorado en Biotecnología Biomédica y Evolutiva

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Carlos Barceló Seguí

Director/a: Dr. Miguel Ángel Miranda Chueca

Director/a: Dra. Bethan V. Purse

Tutor/a: Dr. Carlos Juan Clar

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Dr. Miguel Ángel Miranda Chueca of the University of the Balearic Islands and Dr. Bethan V. Purse of the CEH (Center for Hydrology and Ecology)

WE DECLARE:

That the thesis entitled *Bionomía y modelos de abundancia estacional de las especies del género* Culicoides *(Diptera; Ceratopogonidae) en España, con especial interés en los vectores del virus de la Lengua Azul*, presented by Carlos Barceló Seguí to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document

Signatures

B. Purse

Dr. Miguel Ángel Miranda Chueca Dr. Bethan V. Purse

Palma de Mallorca, 12 September 2019

Als meus pares

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Pareix mentida que ja faci 9 anys que em vaig ficar dins l'increïble món dels insectes. Durant tot el període de la tesi he crescut com a persona, he conegut moltíssima gent i he viatjant molt, per tant, se'm fa difícil recordar-me de totes les persones a les que vull agrair. Començaré per el principi d'aquesta etapa:

La primera persona que vull donar les gràcies es na Marga Miquel, es pot dir que amb ella es amb qui em vaig introduir al món dels vectors de malalties i concretament dels mosquits. Encara record aquell estiu de 2010 cercant larves de moscards a les zones inundades del Pla de Sant Jordi. Va ser tota una aventura i, la veritat, que vaig gaudir molt. A partir d'aquí, vaig sentir molta curiositat per aquest "mundillo" i vaig continuar col·laborant amb tasques del Laboratori de zoologia de la UIB. Allà vaig conèixer als que encara són els meus companys entomòlegs per excel·lència que vull anar citant un per un:

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Listado de abreviaciones

a: Intercept

AgFor: Agro-forestry

AIC: Akaike Information Criterion

BrdMix: Broad leaved forest and mixed forest

CDC: Centre for Disease Control

CEH: Center for Ecology and Hydrology

CGIAR-CSI: Consortium for Spatial Information

CLC: Corine Land Cover

C/t/n: Number of maximum *Culicoides* collected per trap and night

DDaut: Accumulated degree days greater than 10°C in autumn

DDspr: Accumulated degree days greater than 10°C in spring

DDsum: Accumulated degree days greater than 10°C in summer

DDwin: Accumulated degree days greater than 10°C in winter

DIC: Deviance Information Criterion

e.g.: *exempli gratia* (for example)

EEH / EHD: Enfermedad Epizoótica Hemorrágica / Epizootic Haemorrhagic Disease

EEUU: Estados Unidos

EFSA: European Food Safety Authority

Elev.: Elevation

Fam.: Familia

f/fem.: Females

Fig.: Figura / Figure

GLMM: Modelos Mixtos Lineales Generalizados / Generalized Lineal Mixed Models

H': Shannon's diversity index

i.e.: *id est*

IMI: *C. imicola*

K-S: Kolmogórov-Smirnov

K-W: Kruskal-Wallis

L.: Linnaeus

L1: Primer estadio larvario / First instar larvae

L2: Segundo estadio larvario / Second instar larvae

L3: Tercer estadio larvario / Third instar larvae

L4: Cuarto estadio larvario / Fourth instar larvae

LA / BT: Lengua azul / Bluetongue

MCMC: Markov chain Monte-Carlo

MODIS: Moderate Resolution Imaging Spectroradiometer

N / n / #: Number / Sample size

NatGras: Natural Grassland

NDVI: normalized difference vegetation index

NEW: *C. newsteadi*

NF: Hembra nulípara / Nulliparous female

Obs.: Observations

OBS: Obsoletus complex

OW: Overwinter

P: Probability value

PA: Path Analysis models

PastGras: Pasture Grassland

Paut: Precipitation in autumn

pD: Effective number of parameters

PEA / AHS: Peste Equina Africana / African Horse Sickness

PF: Hembra para / Parous female

Phapr: Photoperiod in April

Phmarch: Photoperiod in March

Phnov: Photoperiod in November

Phsep: Photoperiod in September

PIE: Periodo de Incubación Extrínseco

PII: Periodo de Incubación Intrínseco

Pspr: Precipitation in spring

Psum: Precipitation in summer

PUL: *C. pulicaris*

Pwin: Precipitation in winter

RH: Relative Humidity

S.D.: Standard deviation

SB: Schmallenberg

SBV: Schmallenberg Virus

SchVeg: Schlerophyllous Vegetation

SRTM: Shuttle Radar Topography Mission

Subfam.: Subfamilia

SVFP: Seasonal Vector Free Period

UIB: Universidad de las Islas Baleares / University of the Balearic Islands

UV: Ultraviolet

VIF: Variance Inflation Factors

VLA / BTV: Virus de la Lengua azul / Bluetongue virus

W/A: Without abdomen

WND: Wrapped Normal Distribution

μg: Microgramos

ρ: Spearman’s rho correlation coefficient

x̄: Media aritmética / Arithmetic mean

Resumen

Las hembras de varias especies de insectos del género *Culicoides* (Diptera; Ceratopogonidae) transmiten arbovirus que afectan a rumiantes domésticos y salvajes tales como el virus de la Lengua Azul, de la Peste Equina Africana, el virus de Schmallenberg y el virus de la enfermedad hemorrágica epizoótica.

Estos insectos son frecuentemente clasificados por su estado gonotrófico para fines de seguimiento en programas de vigilancia de especies de vectores de la lengua azul. Las hembras paras (PF), las cuales se han alimentado de sangre infectada y han podido replicar el virus a niveles transmisibles, son la única fracción de la población del vector capaz de transmitir el virus de manera eficaz durante una posterior ingesta de sangre sobre un hospedador sano. Por lo tanto, el estudio de la variación estacional de la población de PF resulta de gran interés para evaluar el riesgo de transmisión del virus de la Lengua Azul (VLA) en cada periodo del año. Durante el primer estudio de la presente tesis se utilizaron datos del Programa Nacional de Entomovigilancia de 2008 a 2010 para analizar el patrón estacional de las capturas máximas semanales de hembras nulíparas (NF) y PF de las especies vectores *C. imicola*, complejo Obsoletus, *C. newsteadi* y *C. pulicaris*. Además, se analizó la variación latitudinal del patrón de abundancia estacional de PF en puntos de muestreo que abarcaron un eje Norte-Sur en España continental. Para la mayoría de las especies estudiadas, la abundancia semanal de PF fue siempre más elevada en verano y, excepto en el caso de *C. imicola*, el pico poblacional ocurrió principalmente entre abril y julio lo cual tiene una relación directa con la población capaz de transmitir el VLA en los meses contiguos. El incremento de PF en el caso de *C. imicola* fue de septiembre a noviembre. El análisis de la variación estacional latitudinal de la PF demostró que *C. imicola* no está presente en las provincias del norte mientras que las especies del complejo Obsoletus son las mayoritarias en dichas provincias. Además, se han encontrado provincias en las que hubo periodos del año donde no se capturaron individuos de ninguna especie vector, lo cual se debe tener en cuenta a la hora de calcular el Periodo Estacionalmente Libre de Vectores (SVFP). *Culicoides newsteadi* y *C. pulicaris* estaban igualmente presentes en todas las provincias analizadas mostrando la población más elevada en Toledo, posiblemente debido a su preferencia a las zonas de interior. Estos hallazgos son de gran interés para una mejor comprensión de los periodos de bajo y elevado riesgo de transmisión del VLA en España.

El objetivo del segundo estudio fue analizar la fenología de las hembras adultas de *Culicoides* presentes en España en relación a posibles variables ambientales mediante datos del Plan Nacional de Vigilancia Entomológica de 329 puntos en España desde 2005 hasta 2010 utilizando Modelos Bayesianos Generalizados Lineales Mixtos (GLMM). Se contrastaron efectos climáticos, topográficos, cubierta vegetal y hospedadores sobre la estacionalidad de las hembras adultas. Las especies del complejo Obsoletus fueron las más prevalentes en todos los sitios seguidas de *C. newsteadi*. Las hembras adultas del complejo de Obsoletus fueron las que aparecieron más temprano en la primavera, en un promedio de a principios de abril; mientras que las hembras adultas de *C. imicola* aparecieron en último lugar, en general a principios de julio. Entre las cuatro especies estudiadas, las zonas y años con inviernos cálidos en lugares poco elevados respecto al nivel del mar y con una densidad anual elevada de *Culicoides* hembras adultas, se asociaron con una aparición más temprana y periodos de actividad más largos de estos insectos. En el caso de *C. imicola*, el periodo estacional fue más largo en zonas poco elevadas y más corto en zonas con una elevada acumulación de días con temperaturas sobre los 10ºC y precipitaciones altas durante el invierno. Para las especies del complejo Obsoletus, el periodo de actividad de las hembras adultas también fue más prolongado en zonas poco elevadas con mayor número de horas de sol y temperaturas más cálidas en primavera y otoño, así como en zonas con altas precipitaciones en otoño y gran abundancia de ganado bovino. *Culicoides newsteadi* fue la especie que se vio asociada por una mayor cantidad de variables diferentes. La aparición temprana y largos periodos de actividad de adultos de esta especie se relacionaron con inviernos cálidos, otoños cálidos con altas precipitaciones y áreas agroforestales con vegetación esclerófila y poca pendiente en el terreno. Por otra parte, *C. pulicaris* mostró periodos más largos en sitios con un elevado número de días con temperaturas mayores a 10ºC durante el invierno. Estos resultados demostraron las diferencias ecológicas, biológicas y estacionales entre estas cuatro especies en España, siendo de gran importancia para determinar las zonas con las condiciones ambientales adecuadas para cada especie y evaluar el riego de aparición de brotes de VLA.

En el tercer estudio se utilizaron los mismos datos de vigilancia entomológica de *Culicoides* para realizar modelos de Análisis de Trayectorias (*Path Analysis models*) (PA) con la finalidad de examinar los efectos directos de variables ambientales sobre la estacionalidad de NF y PF de *Culicoides* además de posibles efectos indirectos a través de la abundancia de hembras de estos insectos. Se observó que la abundancia de hembras tiene un papel significativo en la estacionalidad de las cuatro especies de *Culicoides* estudiadas. La cobertura del suelo y la vegetación esclerófila tuvieron un efecto significativamente positivo en el inicio de la actividad de las NF de *C. imicola* a través de la abundancia anual de *Culicoides* hembras; mientras que la acumulación de días sobre los 10ºC en verano tuvo un efecto negativo sobre el final de la actividad de NF y PF de esta especie. Las especies del complejo Obsoletus mostraron que la acumulación de días sobre los 10ºC en primavera tuvo un efecto negativo sobre el inicio de la actividad de NF. Además, la abundancia anual de hembras incrementó significativamente la actividad de los NF y PF del complejo Obsoletus. *Culicoides newsteadi* NF y PF tuvieron un periodo de actividad más largo en zonas con elevada abundancia anual de *Culicoides* hembras. Respecto a *C. pulicaris,* la temperatura en invierno parece ser el factor determinante para esta especie, mostrando una mayor actividad en zonas con mayor acumulación de días sobre los 10ºC en invierno. Estos resultados demostraron que las diferentes especies de *Culicoides* responden a una variable u otra dependiendo de sus requerimientos biológicos ya sean NF o PF.

Para el cuarto estudio se analizó la bionomía en condiciones de laboratorio de especies de *Culicoide*s vectores del VLA y otras especies asociadas a granjas. Se recolectaron hembras grávidas entre primavera y otoño del 2014 en una finca ganadera de Mallorca (España). No había circulación de VLA en ese momento. *Culicoides obsoletus* NF obtenidas en la misma finca fueron utilizadas para alimentarlas con sangre de ganado mediante Hemotek© usando una membrana artificial. Los insectos fueron mantenidos en cajas de cartón y sustrato para la oviposición. Los huevos obtenidos se transfirieron a placas de Petri con medio de gel agar donde las larvas se criaron alimentándolas con nematodos. Las pupas fueron transferidas de nuevo a cajas de cartón hasta la emergencia de los adultos. *Culicoides imicola* fue la especie que tardó más tiempo en ovipositar (8 días); el mayor número de huevos fue depositado por *C. circumscriptus*. Las hembras grávidas de *C. imicola* y del complejo Obsoletus colectadas en el campo mostraron la mayor supervivencia en condiciones de laboratorio comparado con las otras especies incluidas en el presente estudio. El ciclo de vida más largo desde huevo hasta la muerte del adulto, generalmente más de 40 días, fue registrado en la especie *C. cataneii*. Las especies *C. paolae* y *C. circumscriptus* parecieron ser las más adecuadas para la cría en condiciones de laboratorio debido a sus tasas elevadas de oviposición, ciclo de vida corto, elevada supervivencia de la fase adulta y elevado porcentaje de hembras en la progenie. Las especies vectores como *C. obsoletus* fueron difíciles de mantener en el laboratorio debido a sus dificultades para pupar. A pesar de que la tasa de alimentación con sangre registrada de *C. obsoletus* fue de sólo un 6.25%, la oviposición, la esperanza de vida y

el tiempo de eclosión de huevos fueron mayores en las hembras alimentadas artificialmente; mientras que el número de huevos, pupas y adultos emergidos fue mayor en el caso de las hembras grávidas. Este estudio ha contribuido al conocimiento de los parámetros bionómicos básicos de las especies de *Culicoides* vectores y no vectores, destacando las complejidades involucradas en el establecimiento de colonias sostenibles en el laboratorio de especies de *Culicoides* obtenidos en el campo.

Durante el último estudio, también sobre bionomía, se analizó el desarrollo y la duración de la vida de fases juveniles y adultos de *Culicoides* a tres temperaturas diferentes en condiciones de laboratorio de la especie vector *C. obsoletus* así como de otras especies no vectores como *C. circumscriptus*, *C. paolae* y *C. cataneii*. Los insectos fueron recolectados de campo entre la primavera y el otoño de 2015 en dos granjas ganaderas ubicadas en Mallorca (España). Las hembras grávidas se mantuvieron individualmente a 18°C, 25°C y 30°C. Los porcentajes de supervivencia después de la captura fueron bajos (26.1% - 30.7%). La población de *Culicoides* de la granja de *Son Ajaume* mostró el índice de diversidad de Shannon Weaver más elevado. Temperaturas bajas mostraron una mayor tasa de supervivencia de adultos, un tiempo de oviposición más largo y periodos más largos de las fases inmaduras; mientras que las temperaturas elevadas aumentaron el número de huevos, el porcentaje de pupado, la emergencia de adultos y la velocidad de crecimiento de las larvas. Los resultados mostraron que *C. obsoletus* tiene un desarrollo óptimo a 18ºC mientras que *C. circumscriptus* y *C. paolae* parecen ser las especies más adecuadas para el mantenimiento en condiciones de laboratorio a 25ºC y 30ºC. Variaciones en la temperatura/humedad y ensayos con diferentes materiales y substratos para la oviposición deberían tenerse en cuenta en futuros estudios. Entender los requerimientos de las distintas especies de *Culicoides* optimizando los resultados puede ser interesante a la hora de predecir efectos del cambio climático sobre estas especies, además de determinar las condiciones de cría para especies vectores candidatas.

Resum

Les femelles de diverses espècies d'insectes del gènere *Culicoides* (Diptera; Ceratopogonidae) transmeten arbovirus que afecten a remugants domèstics i salvatges com ara el virus de la Llengua Blava, la Pesta Equina Africana, el virus de Schmallenberg i el virus de la malaltia hemorràgica epizoòtica.

Aquests insectes són freqüentment classificats per el seu estat gonotròfic per a fins de seguiment en programes de vigilància d'espècies vectors de la llengua blava. Les femelles pares (PF) que s’han alimentat de sang infectada i han pogut replicar el virus a nivells transmissibles, es l'única fracció de la població del vector que podria transmetre amb eficàcia el virus durant una posterior alimentació sobre un hoste sa. Per tant, l'estudi de la variació estacional de la població de PF resulta de gran interès per avaluar el risc de transmissió del virus de la Llengua Blava (VLA) a cada període de l'any. Durant el primer estudi de la present tesi es van utilitzar dades del Programa Nacional de Entomovigilancia de 2008 al 2010 per analitzar el patró estacional de les captures màximes setmanals de femelles nul·lípares (NF) i PF de les espècies vectors *C. imicola*, complex Obsoletus, *C. newsteadi* i *C. pulicaris*. A més, es va analitzar la variació latitudinal del patró d’abundància estacional de PF a punts de mostreig que seguien un eix Nord-Sud dins l’Espanya continental. Per a la majoria de les espècies estudiades, l’abundància setmanal de PF va ser sempre més elevades a l'estiu i, excepte en el cas de *C. imicola*, el pic estacional de PF va ocórrer principalment entre abril i juliol la qual cosa té una relació directa amb la població capaç de transmetre el VLA en els mesos següents. L'increment de PF en el cas de *C. imicola* va ser de setembre a novembre. L'anàlisi de la variació estacional latitudinal de la PF va demostrar que a les províncies del nord d’Espanya *C. imicola* no esta present mentre que les espècies del complex Obsoletus son les espècies majoritàries a aquestes províncies. A més, s’han trobat províncies on hi va haver períodes de l'any on no es va capturar cap individu de les espècies vectors de LA, la qual cosa s'ha de tenir en compte a l'hora de calcular el Període Estacionalment Lliure de Vectors (SVFP). *Culicoides newsteadi* i *C. pulicaris* foren igualment presents a totes les províncies analitzades mostrant la població més elevada a Toledo, possiblement a causa de la preferència d’aquestes espècies per a les zones d'interior. Aquestes troballes són de gran interès per a una millor comprensió dels períodes de baix i elevat risc de transmissió del VLA a Espanya.

L'objectiu del segon estudi es va ser analitzar la fenologia de les femelles adultes de *Culicoides* presents a Espanya en relació a possibles variables ambientals mitjançant dades del Pla Nacional de Vigilància Entomològica de 329 punts a Espanya des de 2005 fins 2010 emprant Models Bayesians Generalitzats Lineals Mixts (GLMM). Es van contrastar efectes climàtics, topogràfics, coberta vegetal i hostes sobre l’estacionalitat de les femelles adultes. Les espècies del complex Obsoletus foren les més prevalents en tots els llocs seguides de *C. newsteadi*. Les femelles adultes del complex Obsoletus van ser les que van aparèixer més prompte a la primavera, en una mitjana de a principis d'abril; mentre que les femelles adultes de *C. imicola* van aparèixer en últim lloc, en general a principis de juliol. Entre les quatre espècies estudiades, els llocs i anys amb hiverns càlids seguits per zones poc elevades respecte al nivell del mar i una elevada densitat anual de *Culicoides* femelles adultes, es van associar amb una aparició més primerenca i períodes d'activitat més llargs d'aquests insectes. En el cas de *C. imicola*, el període estacional va ser més llarg en zones poc elevades y més curt a zones amb major acumulació de dies amb temperatures sobre els 10ºC i precipitacions elevades durant l'hivern. Per a les espècies del complex Obsoletus, el període d'activitat de les femelles adultes també va ser més perllongat en zones poc elevades amb major nombre d’hores de sol i temperatures mitjanes més càlides a la primavera i a la tardor; així com en zones amb altes precipitacions a la tardor i gran abundància de bestiar boví. *Culicoides newsteadi* va ser l’espècie que es va veure associada amb una major quantitat de variables diferents. Una aparició primerenca i llargs períodes d'activitat d’adults d'aquesta espècie es van relacionar amb hiverns càlids, tardors càlides amb altes precipitacions i àrees agroforestals amb vegetació escleròfil·la i poca pendent en el terreny. D'altra banda, *C. pulicaris* va mostrar períodes més llargs dels adults en llocs amb alta acumulació de dies sobre els 10ºC durant l'hivern. Aquests resultats van demostrar les diferències ecològiques, biològiques i estacionals entre aquestes quatre espècies a Espanya, sent de gran importància per determinar les zones amb les condicions ambientals adequades per a cada espècie i avaluar el risc d’aparició de brots de VLA.

En el tercer estudi vam fer servir les mateixes dades de vigilància entomològica de *Culicoides* per realitzar models de Anàlisi de Trajectòries (*Path Analysis models*) amb la finalitat d'examinar els efectes directes de variables ambientals sobre l'estacionalitat de NF i PF de *Culicoides* a més de possibles efectes indirectes a través de l'abundància de femelles d'aquests insectes. Es va observar que l’abundància de femelles tenen un paper significatiu en la estacionalitat de les quatre especies de *Culicoides* estudiades. La cobertura del sòl com la vegetació escleròfil·la van tenir un efecte significativament positiu en l'inici de l'activitat de les NF de *C. imicola* a través de l'abundància anual de *Culicoides* femelles; mentre que l'acumulació de dies sobre els 10ºC a l'estiu va tenir un efecte negatiu sobre el final de l'activitat de NF i PF d'aquesta espècie. Les espècies del complex Obsoletus van mostrar que l'acumulació de dies sobre els 10ºC a la primavera va tenir un efecte negatiu sobre l'inici de l'activitat de NF. A més, l'abundància anual de femelles va incrementar significativament l'activitat dels NF i PF del complex Obsoletus. *Culicoides newsteadi* NF i PF van tenir un període d'activitat més llarg en zones amb elevada abundància anual de *Culicoides* femelles. Pel que fa a *C. pulicaris*, la temperatura a l'hivern sembla ser el factor determinant per a aquesta espècie, mostrant una major activitat en zones amb major acumulació de dies sobre els 10ºC a l'hivern. Els resultats van demostrar que les diferents espècies de *Culicoides* responen a una variable o una altra depenent dels seus requeriments biològics ja siguin NF o PF.

Per al quart estudi es va estudiar la bionomia en condicions de laboratori de les espècies de *Culicoides* vectors del VLA i altres espècies associades a granges, subjectes a condicions de laboratori. Es van recol·lectar femelles gràvides entre primavera i tardor del 2014 a una finca ramadera de Mallorca (Espanya). No hi havia circulació de VLA en aquell moment. *Culicoides obsoletus* NF obtingudes a la mateixa finca van ser utilitzades per alimentar-les amb sang de bestiar mitjançant Hemotek© a través d’una membrana artificial. Els insectes foren mantinguts en caixes de cartró i substrat per a la oviposició. Els ous obtinguts es van transferir a plaques de Petri amb medi de gel agar on les larves es van criar alimentant-les amb nematodes. Les pupes van ser transferides de nou a caixes de cartró fins a l'emergència dels adults. *Culicoides imicola* va ser l'espècie que va trigar més temps en ovipositar (8 dies); el major nombre d'ous va ser dipositat per *C. circumscriptus*. Les femelles gràvides de *C. imicola* i del complex Obsoletus recol·lectades al camp van mostrar la major supervivència d’adults en condicions de laboratori comparat amb les altres espècies incloses en el present estudi. El cicle de vida més llarg des de ou fins la mort de l’adult, generalment més de 40 dies, va ser registrat en l'espècie *C. cataneii*. Les espècies *C. paolae* i *C. circumscriptus* van semblar ser les més adequades per a la cria en condicions de laboratori degut a les seves elevades taxes d‘oviposició, cicle de vida curt, elevada supervivència de la fase adulta i elevat percentatge de femelles en la progènie. Les espècies vectors com *C. obsoletus* van ser difícils de mantenir en el laboratori degut a la seva dificultat per pupar. Tot i que la taxa d'alimentació registrada per *C. obsoletus* va ser de només un 6.25%, la oviposició, l'esperança de vida i el temps d'eclosió d'ous van ser majors en les femelles alimentades artificialment; mentre que el nombre d'ous, pupes i adults emergits va ser més gran en el cas de les femelles gràvides. Aquest estudi ha contribuït al coneixement dels paràmetres bionòmics bàsics de les espècies de *Culicoides* vectors i no vectors, destacant les complexitats involucrades en l'establiment de colònies en el laboratori d'espècies de *Culicoides* obtingudes al camp.

Durant l'últim estudi, també sobre bionomia es va analitzar el desenvolupament i la durada de la vida de fases juvenils i adults de *Culicoides* a tres temperatures diferents en condicions de

laboratori de l'espècie vector *C. obsoletus* així com d'altres espècies no vectors com *C. circumscriptus*, *C. paolae* i *C. cataneii*. Els insectes van ser recol·lectats al camp entre la primavera i la tardor de 2015 a dues granges ramaderes ubicades a Mallorca (Espanya). Les femelles gràvides es van mantenir individualment a 18°C, 25°C i 30°C. Els percentatges de supervivència després de la captura van ser baixos (26.1% - 30.7%). La població de *Culicoides* de la granja de *Son Ajaume* va mostrar l'índex de diversitat de Shannon Weaver més elevat. Temperatures baixes van augmentar la taxa supervivència dels adults i el temps de oviposició, a més d'un augment dels períodes de les fases juvenils; mentre que temperatures elevades van augmentar el nombre d'ous, el percentatge de pupat, l'emergència d'adults i la velocitat del creixement larvari. Els resultats van mostrar que *C. obsoletus* te el seu òptim desenvolupament a 18ºC, mentre que *C. circumscriptus* i *C. paolae* semblen ser les espècies més adequades per al manteniment en condicions de laboratori a 25ºC i 30ºC. Variacions en la temperatura/humitat i assajos amb diferents materials i substrats per a la oviposició s'haurien de tenir en compte en futurs estudis. Entendre els requeriments de les diferents espècies de *Culicoide*s optimitzant els resultats pot ser interessant a l'hora de preveure els efectes del canvi climàtic sobre aquestes especies, a més de determinar les condicions de cria per especies vectors candidates.

Abstract

Females of several biting midges species from genus *Culicoides* (Diptera; Ceratopogonidae) transmit arboviruses affecting wild and domestic ruminants such as Bluetongue, African Horse sickness, Schmallenberg virus and Epizootic Haemorrhagic disease viruses.

These insects are frequently age graded for monitoring purposes in surveillance programs of bluetongue vector species. Parous females (PF) that has taken an infected blood meal and could have replicated the virus to transmissible levels are the only fraction of the entire population of vector species that could effectively transmit viruses in a subsequent blood meal. The study of the seasonal variation of PF would be of great interest for evaluating the risk of transmission of Bluetongue virus (BTV) through each period of the year. During the first study of the current thesis, data of the Spanish Entomosurveillance National Program from 2008 to 2010 were used to analyse the seasonal pattern of the maximum weekly catches of nulliparous females (NF) and PF of the potential vector species *C. imicola*, Obsoletus complex, *C. newsteadi*, and *C. pulicaris*. In addition, latitude variation on the seasonal abundance patterns of PF has been analysed in trap sites spanning a North-South axis in

mainland Spain. For most of the studied taxa, the weekly abundance of PF was always highest in summer and, except for *C. imicola*, the peak of abundance mainly occurred between April and July. This pattern has a direct relationship with the population capable of transmitting the BTV in the following months. The highest abundance of PF in case of *C. imicola* was from September to November. The analysis of the latitudinal seasonal variation of PF in Spain has demonstrated that Northern provinces showed absence of *C. imicola* during the whole year while the Obsoletus species were more present in Northern areas. In fact, depending on the province and the species, there were periods of the year were no individuals of any vector species were collected, which should be taken into account in order to calculate the Seasonally Vector-Free Period (SVFP). *Culicoides newsteadi* and *C. pulicaris* were equally present in all analysed provinces showing the highest population in Toledo, probably related to their inland preferences. These findings would be of interest for a better understanding of the periods of low and high risk of transmission of BTV in Spain.

The objective of the second study of this thesis was to analyse the adult female phenology of the vector species of *Culicoides* present in Spain in relation to potential environmental drivers using a large vector surveillance dataset from 329 sites in Spain from 2005 to 2010 and Bayesian Generalised Linear Mixed Model (GLMM) approaches. The effects of climate, topographic, land-cover and host factors on adult female seasonality were contrasted. Obsoletus complex species were the most prevalent across sites followed by *C. newsteadi*. Obsoletus complex adult females appeared earliest in spring amongst taxa studied, on average in early April, whereas *C. imicola* adult females appeared last, on average in early July. For all taxa studied, sites and years with warm winters, that were at low elevation from the sea level and had high annual adult female population size, were associated with earlier appearance of adult females and longer adult female seasons. For *C. imicola*, the length of the female adult season was longest in sites at low elevation and shortest with more accumulated degree days over 10ºC and higher rainfall during winter. For Obsoletus complex species, female adult seasons were also longest in sites at low elevation, with long spring and autumn daylight hours and warmer spring and autumn average temperatures as well as in sites with high precipitation in autumn and high abundance of cattle. *Culicoides newsteadi* was the taxa associated with many different variables. Early appearance and long adult seasons of this species were found in sites with warm winters and autumns and high precipitation in autumn, and in sites with higher coverage of agro-forestry with schlerophyllous vegetation and gently sloping land. Meanwhile, *C. pulicaris* showed longer adult periods in sites with high number of accumulated degree days over 10ºC during winter. These results demonstrated the eco-climatic, biology, seasonal and behaviour differences among these four taxa in Spain, which is an important issue to determine the sites with suitable environmental circumstances for each particular species and assess the risk of BTV outbreaks.

In the third study we used the same *Culicoides* surveillance data to perform Path Analysis (PA) models to examine the direct effects of environmental variables on the seasonal metrics of *Culicoides* NF and PF adults versus indirect effects of site abundance of *Culicoides* female on seasonality. Female abundance plays a significant role in the seasonality of the four *Culicoides* species studied. Land cover such as schlerophyllous vegetation had a significant positive effect on the start of the season timing of *C. imicola* NF via *Culicoides* female abundance. Accumulated degree days over 10ºC in summer had a significant negative effect on the end of season timing of NF and PF of this species. Obsoletus complex species showed that accumulated degree days over 10ºC in spring had a negative significant effect on the start of the season timing of NF of this species. The annual females abundance increased significantly the activity period of NF and PF of Obsoletus complex. *Culicoides newsteadi* NF and PF showed longest periods in sites with higher annual *Culicoides* female abundance. Regarding *C. pulicaris*, temperature during winter seems to be a determinant driver for that species, being more active in sites with highest accumulated degree days over 10ºC in winter. The results showed that the different species of *Culicoides*, NF or PF, respond to one variable or another depending on their biological requirements.

The fourth study investigates the bionomics of farm-associated *Culicoides* vector species under laboratory conditions. Gravid females were collected from the field from spring to autumn 2014 on a cattle farm in Majorca (Spain). No BTV was circulating at that time. *Culicoides obsoletus* NF were engorged using cattle blood in a Hemotek© with artificial membrane. Insects were kept individually in cardboard boxes with moistened cotton wool and filter paper as a substrate for oviposition. Eggs were transferred to Petri dishes with agar gel medium and larvae were reared in the agar fed with nematodes. Pupae were transferred again to cardboard boxes till adults emerged. *Culicoides imicola* took the longest time to oviposit (8 days); the highest number of eggs was laid by *C. circumscriptus*. Field collected gravid *C. imicola* and Obsoletus complex females showed the longest lifespan among the species included in the current study. The longest life-cycle (from egg to death of adult), generally more than 40 days, was recorded from *C. cataneii*. *Culicoides paolae* and *C. circumscriptus* seemed to be the most suitable for laboratory rearing due to their high oviposition rates, short life-cycle, long adult lifespan and female biased sex ratio. Vector species such as *C. obsoletus* appeared difficult to colonise in the laboratory due to their pupation difficulties. The blood-feeding rate recorded by *C. obsoletus* was only 6.25%, however, oviposition, lifespan and time to hatch was higher in artificial lab-engorged females; whereas the number of eggs, pupae and adults emerged were higher in case of the field gravid females. This research contributes to the knowledge of the basic bionomic parameters of vector and non-vector *Culicoides* species, highlighting the complexities involved in the establishment of laboratory colonies of field collected *Culicoides* species.

For the last study, also related to bionomics, we analysed the *Culicoides* sub-adult development and lifespan at three different temperatures in laboratory conditions of the vector species *C. obsoletus* as well as the non-vector species *C. circumscriptus*, *C. paolae* and *C. cataneii*. Insects were collected from field between spring and autumn 2015 in two cattle farms located in Majorca (Spain). Gravid females were kept individually at 18°C, 25°C and 30°C. The percentages of survival after capture were low (26.1% - 30.7%). *Culicoides* population from *Son Ajaume* farm showed the highest Shannon Weaver diversity index. Low temperatures increased the adult lifespan, time to oviposit and life-cycle development whereas high temperatures increased the number of eggs, percentages of pupation and adult emergence as well as the larvae growth rate. The results showed that *C. obsoletus* seemed to have optimum development at 18ºC whereas *C. circumscriptus* and *C. paolae* were the most suitable species for maintaining in laboratory conditions at 25ºC and 30ºC. Variations in temperature/humidity and assays with different materials and substrates for oviposition should be taken into account in future studies. Understand the requirements of the different species of *Culicoides* optimizing the results should be of special interest for predict environmental change effects on these species, in addition to determine the rearing conditions for candidate *Culicoides* vectors.

1. Introducción general

1.1. Las enfermedades transmitidas por vectores

Las enfermedades transmitidas por artrópodos ("Arthropod-borne diseases") han ido aumentando considerablemente en diversas partes del mundo debido al cambio climático y otros factores antropogénicos (Jamison *et al*. 2015, Carvalho *et al*. 2016, Samy y Peterson 2016). Existen unas 500 especies de arbovirus (virus transmitidos por artrópodos), entre ellos el virus de la Fiebre Amarilla, el Dengue o la Encefalitis Equina del Este que afectan a humanos; otros que solo afectan a animales como el virus de la Lengua Azul y la Peste Equina Africana y otros que son zoonóticos, es decir, causan enfermedades tanto en humanos como animales como el virus de la Fiebre del Valle del Rift o el virus del Nilo Occidental (Mellor 2000, Ostfeld y Keesing 2000).

Dentro de los vectores biológicos de arbovirosis, podemos encontrar diversos taxones, siendo cuatro los más importantes: las garrapatas (Fam. Ixodidae), los mosquitos (Fam. Culicidae), los flebótomos (Subfam. Phlebotominae) y finalmente los jejenes (*Culicoides* spp.).

1.2. El género *Culicoides*

Los *Culicoides* son un género de dípteros compuesto por unas 1357 especies incluidas dentro de la familia Ceratopogonidae (Borkent 2015). No tienen un nombre común concreto en España, en algunos lugares les llaman jejenes o beatillas mientras que en países anglosajones les llaman "biting midges" o "punkies". La importancia de este género radica principalmente en su papel en el sector médico-veterinario ya que se trata de insectos hematófagos capaces de transmitir arbovirus y otros patógenos a animales salvajes y domésticos, siendo el virus de la Lengua Azul (VLA) uno de los más conocidos en Europa.

1.2.1. Posición Taxonómica

Los ceratopogónidos se dividen en cuatro subfamilias: por una parte Ceratopogoninae donde se incluye el género *Culicoides*, y por otro lado las subfamilias Leptoconopinae, Forcipomyiinae y Dasyheleinae donde se incluyen otros géneros hematófagos como *Forcipomyia* Meigen, *Atrichopogon* Kieffer, *Leptoconops* Skuse, *Kolenohelea* Kieffer, *Dasyhelea* Kieffer, *Ceratopogon* Meigen, *Allohelea* Kieffer y *Shizohelea* Kieffer. Por lo tanto, la posición taxonómica del género del presente trabajo sería la siguiente:

Reino: Animalia
- Subreino: Eumetazoa
  - Filo: Arthropoda
    - Clase: Hexapoda
      - Subclase: Insecta
        - Orden: Diptera
          - Suborden: Nematocera
            - Infraorden: Culicomorpha
              - Superfamilia: Chironomoidea
                - Familia: Ceratopogonidae
                  - Subfamilia: Ceratopogoninae
                    - Tribu: Culicoidini
                      - Género: *Culicoides*

1.2.2. Distribución geográfica y hábitat

De los 103 géneros de ceratopogónidos, el género *Culicoides* es el más rico en especies hasta el momento. Se encuentran distribuidos por todo el planeta entre las regiones tropicales y la tundra excepto Nueva Zelanda, la Antártida, Hawái y Patagonia (Borkent y Wirth 1997, Mellor y Leake 2000, Meiswinkel *et al*. 2004c). Se conocen 117 especies de *Culicoides* en Europa de las cuales 82 han sido descritas en España (Rawlings 1996, Sarto I Monteys *et al*. 2009, Alarcón-Elbal y Lucientes 2012, Sánchez-Murillo *et al*. 2015).

Estos insectos son particularmente abundantes en granjas donde se encuentran sus hospedadores (Tabachnick 2004). Sus larvas crían principalmente en suelos ricos en materia orgánica (Bravermann *et al*. 1974, Mellor y Pitzolis 1979, Foxi y Delrio 2010, Harrup *et al*. 2013, Zimmer *et al*. 2014b, Purse *et al*. 2015) en una gran variedad de hábitats, dependiendo de la especie, desde las “highlands” (tierras altas) en Escocia y zonas turísticas, hasta campos de golf en el caso de *Culicoides impunctatus* Goethebuer (Blackwell *et al*. 1994, 1999) y también en orillas de estanques o zonas irrigadas en el caso de *Culicoides sonorensis* Wirth and Jones (Mayo *et al*. 2014).

1.2.3. Características del género *Culicoides*

1.2.3.1. Morfología

Huevos: Son largados en forma de banana de unos 400 µg de largo por 50 µg de ancho de color blanquinoso que se van oscureciendo con el contacto con el aire (Mellor *et al*. 2000). La superficie de los huevos suele tener una ornamentación característica (Fig. 1).

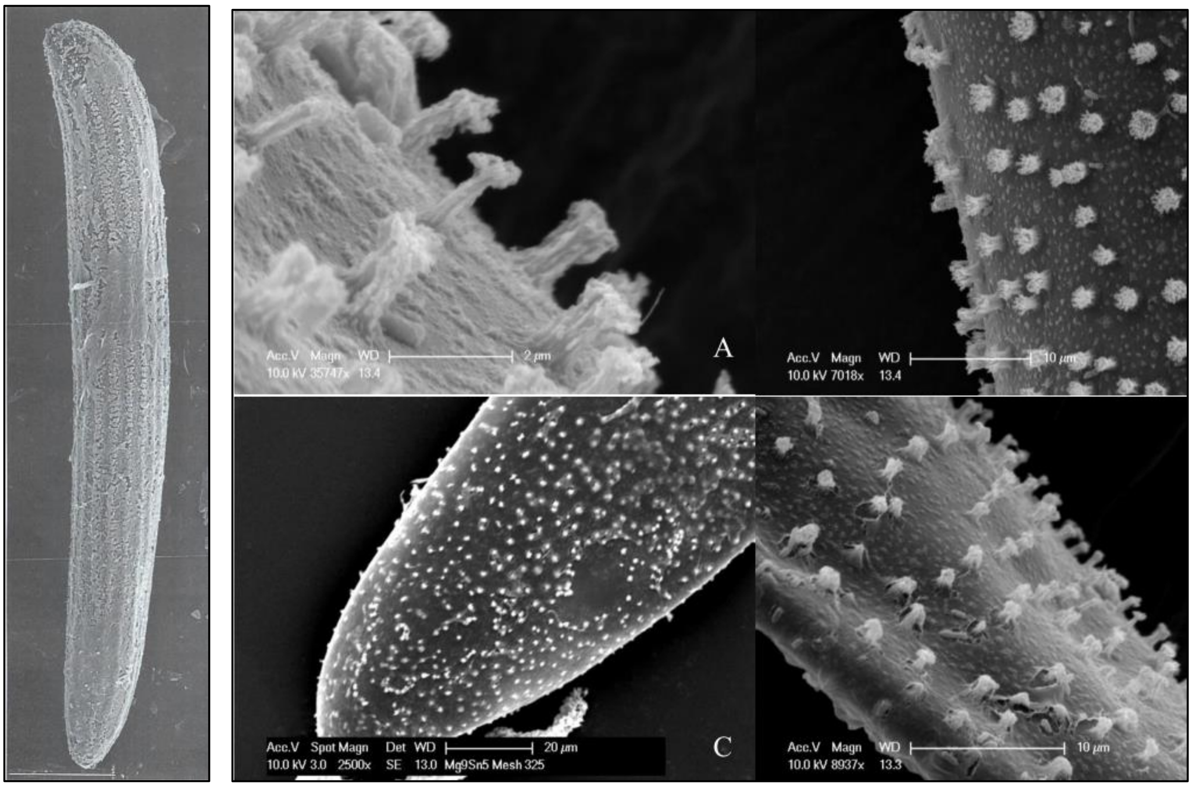


Figura 1. Fotografías con microscopio electrónico de barrido. Izquierda: Vista lateral de un huevo de *Culicoides circumscriptus* Kieffer. Escala 50 µg. Fuente: Day *et al*. (1997). Derecha: Detalle de la ornamentación de la superficie del huevo de *C. sonorensis*. Distintas escalas. Fuente: Abubekerov (2014) y Abubekerov y Mullens (2017).

Larvas: Tienen 4 estadios larvarios ápodos de aspecto vermiforme (Mellor *et al*. 2000, González y Goldarazena 2011). La cápsula cefálica está fuertemente esclerosada, posee quetas simples (Fig. 3) y tiene forma triangular variando de color entre amarillo ámbar y marrón con un collar post occipital de color marrón oscuro. Los ojos tienen forma de coma (Murphree y Mullen 1991, Slama *et al*. 2013, Abubekerov 2014) y el aparato bucal está bien desarrollado con labro, maxilas, mandíbulas y faringe (hipostoma, hipofaringe y epifaringe) (Fig. 2, A).

El tórax está dividido en tres regiones protórax, mesotórax y metatórax que presentan pigmentación amarillenta y marronosa (Fig. 2, A). La región abdominal está formada por nueve segmentos y es de color blanquinosa con presencia de quetas en la región caudal (Slama *et al*. 2013) (Figs. 2, B y 4).

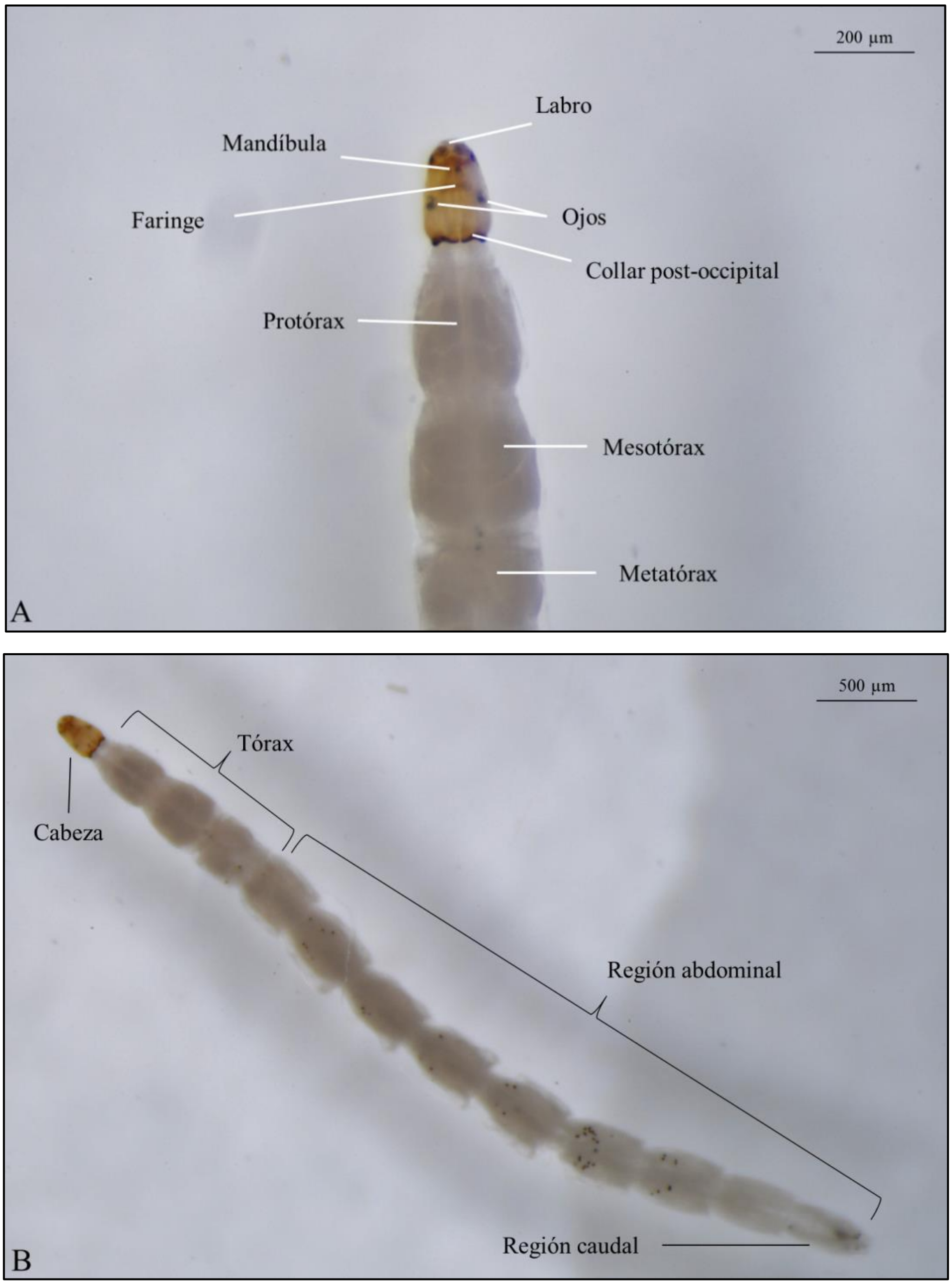


Figura 2. Fotografías realizadas con lupa binocular del detalle de la región cefálica y tórax de una larva L4 de *Culicoides paolae* Boorman (A). Detalle del cuerpo entero de la larva donde se observan los segmentos abdominales (B). Fuente: C. Barceló.

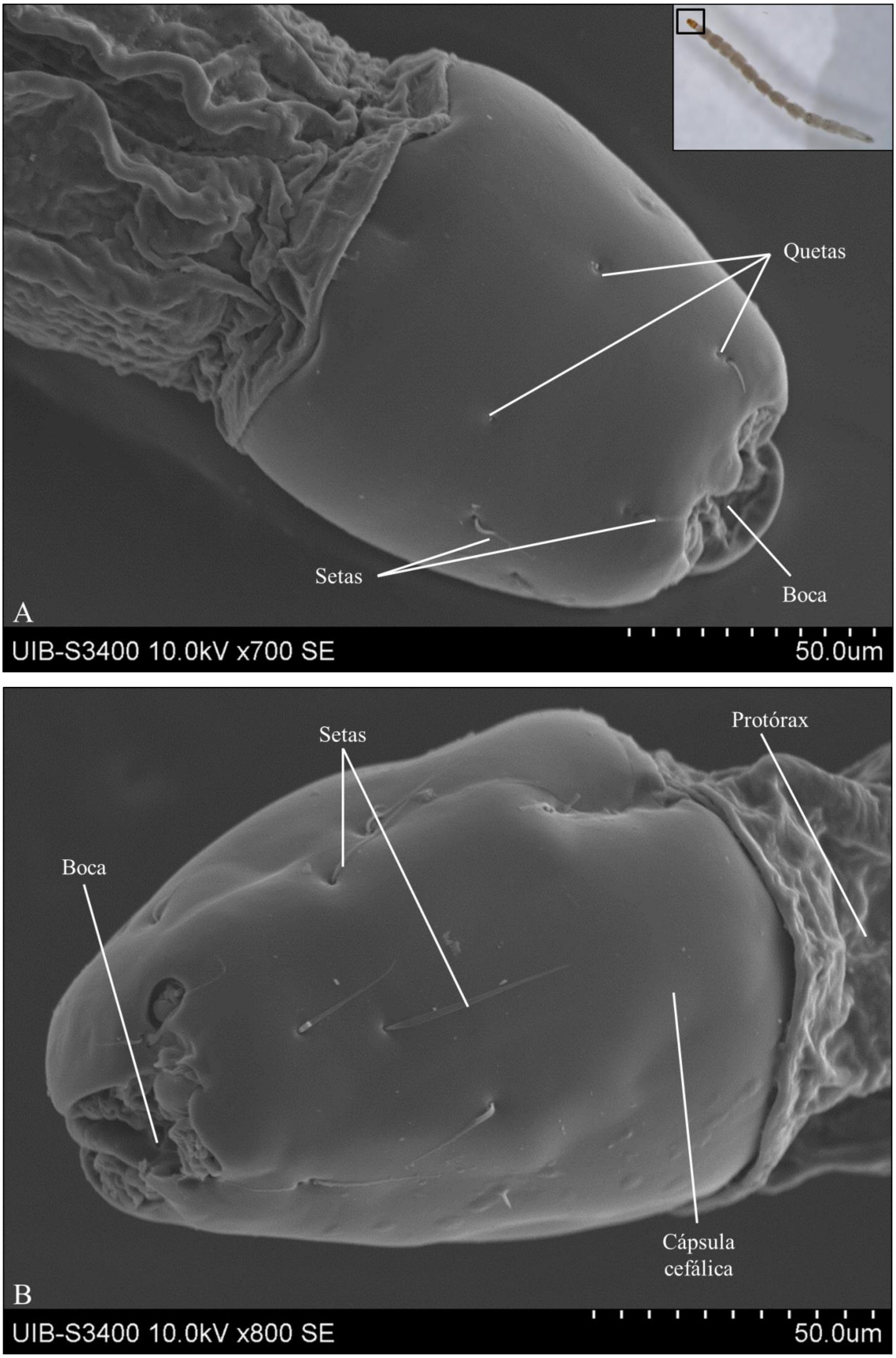


Figura 3. Detalle de la cabeza de una larva L4 de *Culicoides obsoletus* (Meigen) realizada con microscopio electrónico de barrido. Vista ventral (A) y vista latero-ventral (B). Fuente: C. Barceló.

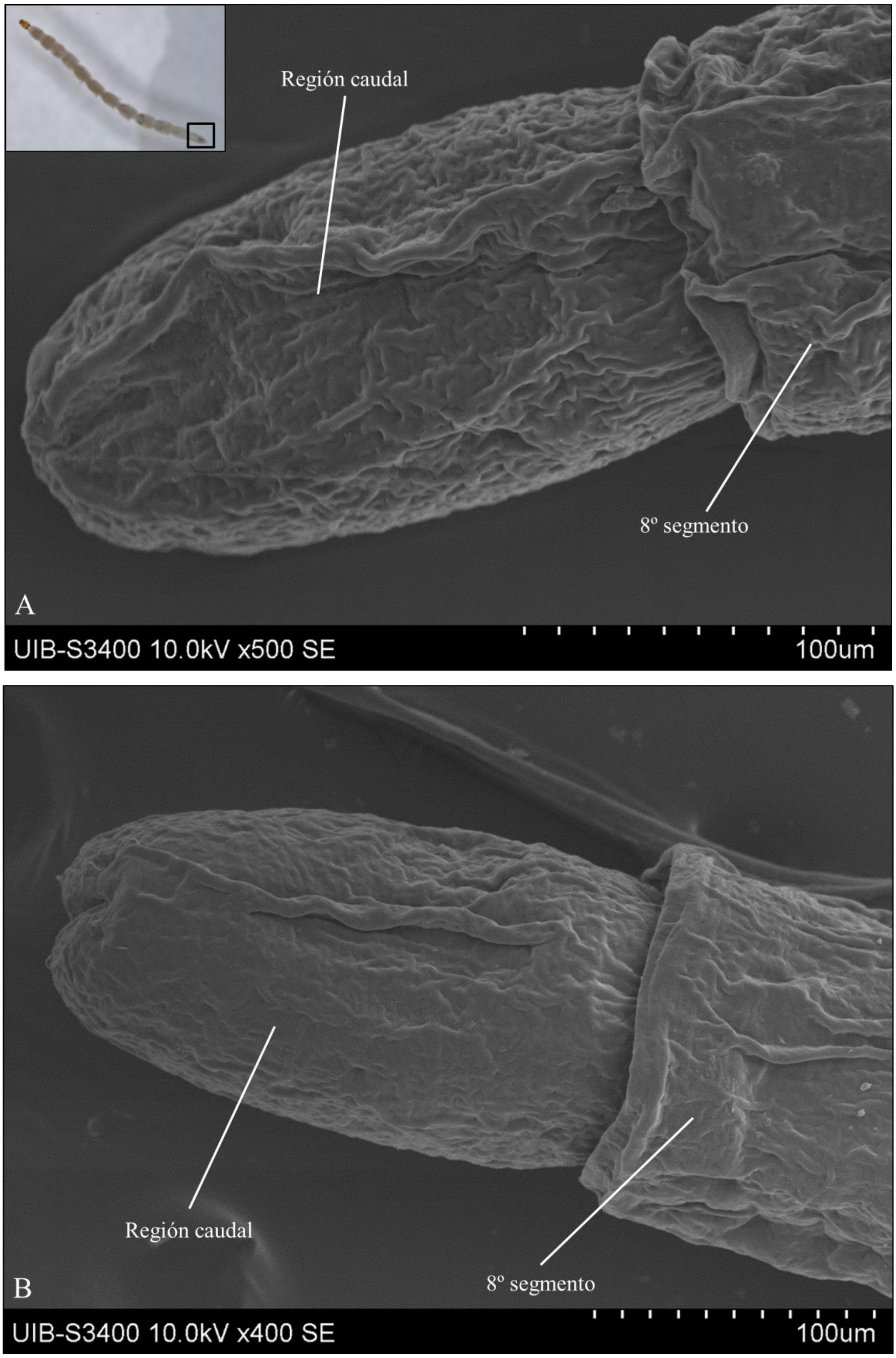


Figura 4. Detalle de la región caudal de una larva L4 de *C. obsoletus* realizada con microscopio electrónico de barrido. Vista dorsal (A) y vista ventral (B). Fuente: C. Barceló.

Pupa: De tipo obteca típica de los nematóceros. Es de color marrón amarillento con partes más oscuras y cubierta de espinas, quetas, sensilias y protuberancias (Holbrook 1996). Posee dos cornetes respiratorios dorsales insertados en el protórax. La cabeza tiene un apotoma dorsal con espinas y escleritos dorso-laterales (Fig. 5). Los cornetes respiratorios están provistos de poros localizados en distintos lugares dependiendo de la especie (Shults *et al*. 2016) (Fig. 6). En la parte ventral se pueden distinguir las partes del futuro imago (patas, antenas, ojos, etc.) (Fig. 5, B) y poseen dimorfismo sexual en el segmento 9 donde los machos presentan lóbulo genital (Shults *et al*. 2016) (Fig. 7).

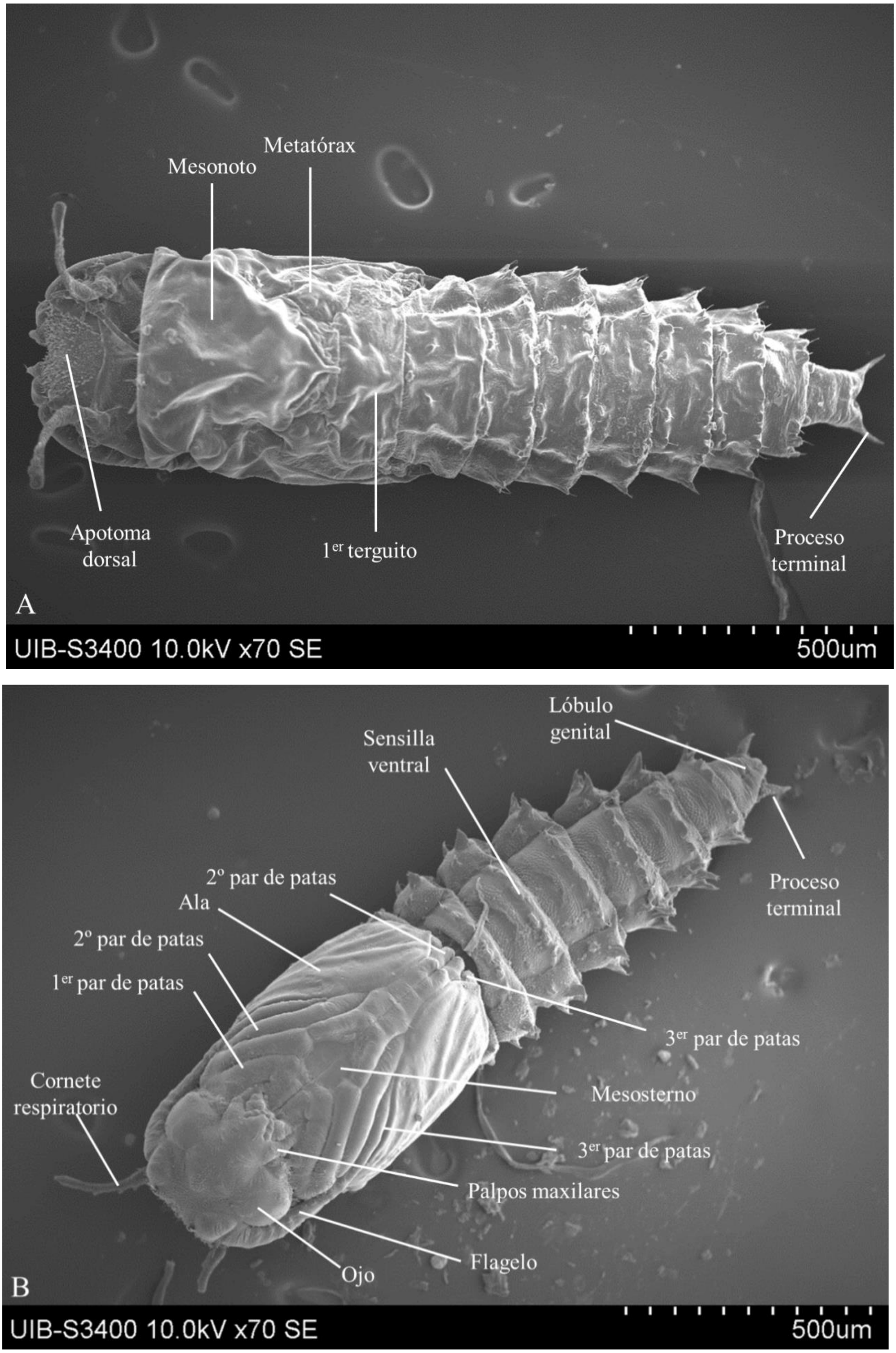
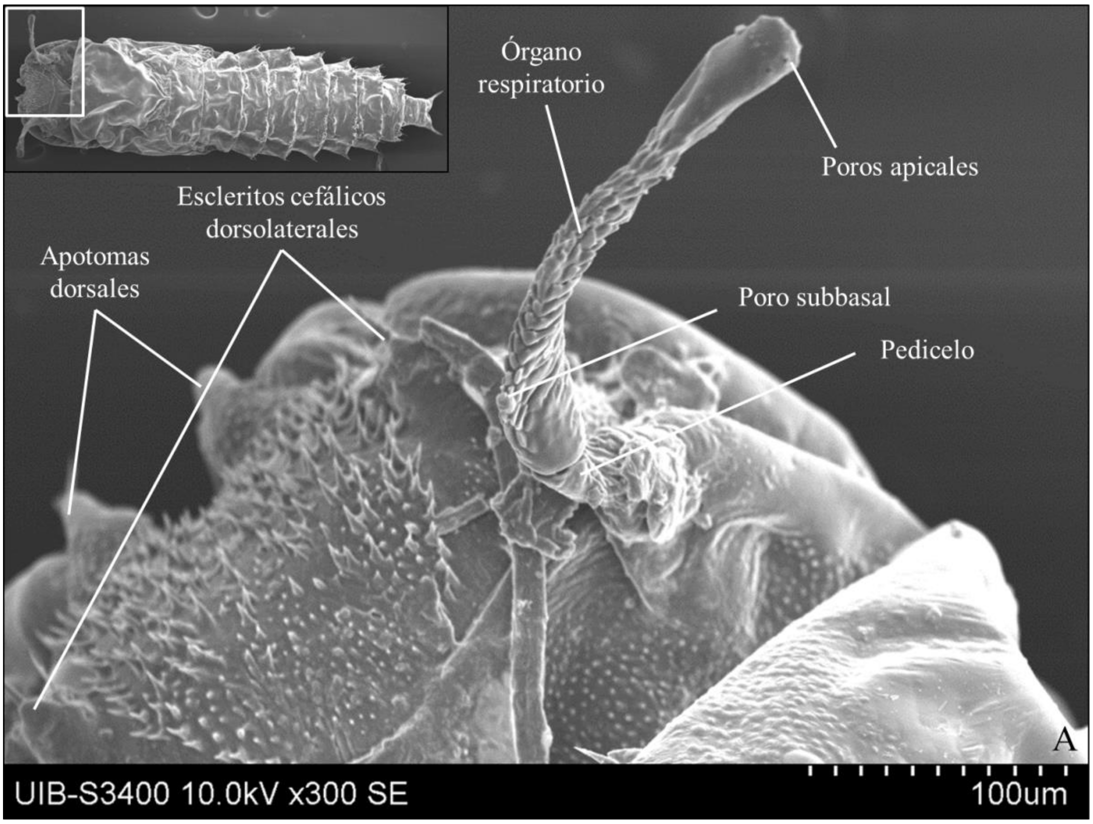


Figura 5. Vista dorsal de una pupa de *C. circumscriptus* (A) y vista ventral de una pupa de *C. paolae* macho (B) realizadas con microscopio electrónico de barrido. Fuente: C. Barceló.



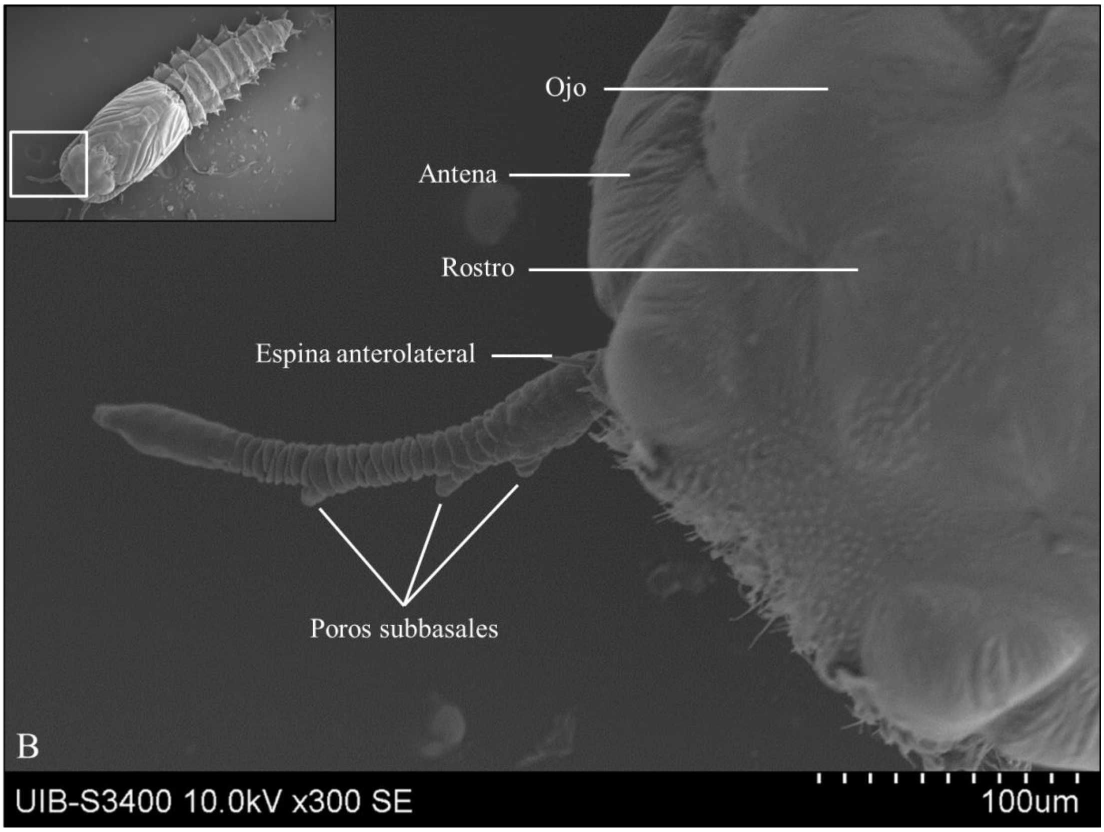


Figura 6. Detalle de la región cefálica de la pupa donde se aprecian los cornetes respiratorios y la localización de los poros realizada con microscopio electrónico de barrido. Vista dorsal de *C. circumscriptus* (A) y vista ventral de *C. paolae* (B). Fuente: C. Barceló.

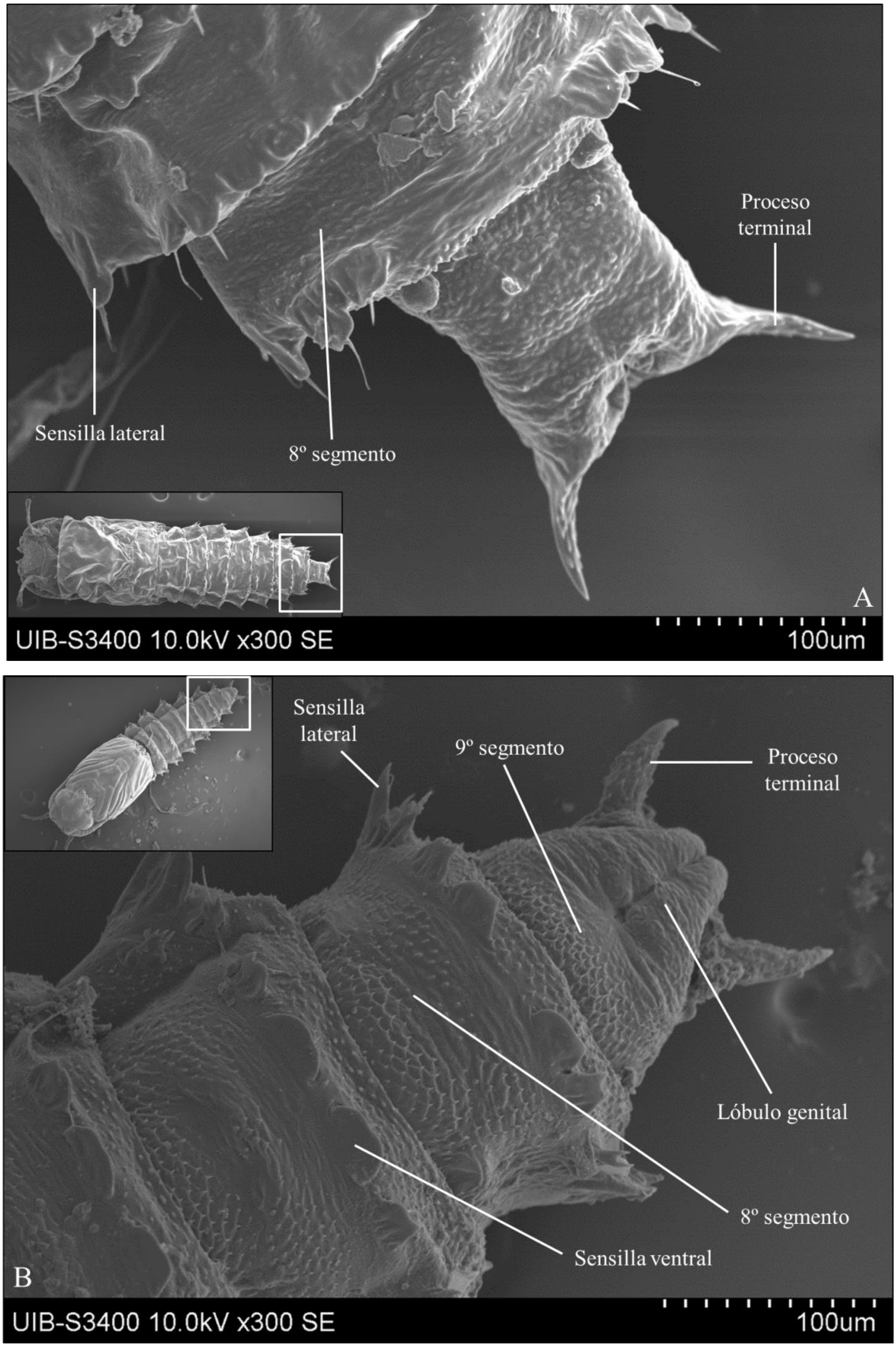


Figura 7. Detalle del lóbulo genital de una pupa donde se aprecia el dimorfismo sexual. Hembra de *C. circumscriptus* (A) y macho de *C. paolae* (B). Fuente: C. Barceló.

Adultos (imago): Se trata de insectos de pequeño tamaño entre 1-5 mm de longitud dependiendo de la especie y con un peso que alcanza los 0.5 µg (González y Goldarazena 2011) (Fig. 8). Su cuerpo es compacto con las alas plegadas una encima de la otra sobre el abdomen cuando el insecto está en reposo.

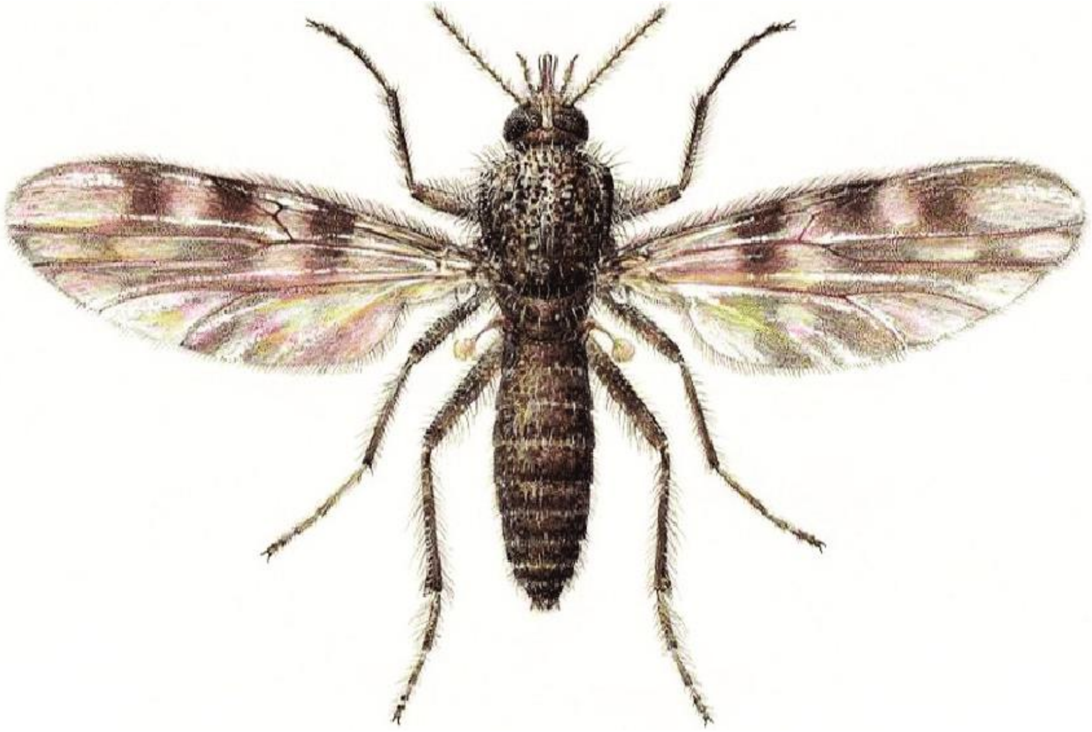


Figura 8. Aspecto general de un *Culicoides* adulto, en este caso, *C. impunctatus*. Se puede apreciar el patrón de manchas alares característico de esta especie. (Diseño de Carim Nahaboo).

- Cabeza: Posee dos ojos compuestos de gran tamaño que no presentan pilosidades (Konurbayev 1965); un par de antenas compuestas por quince segmentos cubiertos de sensilas que tienen carácter taxonómico (Ronderos 1999). Los machos tienen las antenas plumosas (Fig. 9) mientras que las hembras las tienen filiformes. Aparato bucal telmófago (tipo cortador-chupador) con dos palpos maxilares; mandíbulas afiladas y maxilas estiliformes (Delécolle 1985). El macho tiene el aparato bucal reducido ya que no se alimentan de sangre. Estas características pueden cambiar en individuos intersexo donde se mezclan características de los dos sexos en un mismo individuo, como ocurre muy frecuentemente en la especie *C. circumscriptus* (Sánchez-Murillo *et al*. 2011).

- Tórax: Posee un par de alas con venación característica del género y un patrón de manchas que permite diferenciar gran parte de las especies de *Culicoides* (Meiswinkel *et al*. 2004c). Las patas tienen peines tibiales también de valor taxonómico, las uñas de los tarsos son simples en las hembras y dividida en los machos.
- Abdomen: Está formado por nueve segmentos. La genitalia de las hembras posee entre una y tres espermatecas mientras que los machos presentan un abdomen más estrecho y alargado terminado en estructuras prominentes de valor taxonómico (Campbell y Pelham-Clinton 1960, Nielsen y Kristensen 2011) (Fig. 9). Como se ha comentado anteriormente, puede variar en los individuos intersexo. Tanto las espermatecas como la genitalia son importantes como rasgo taxonómico.

Figura 9. Macho de *C. obsoletus* realizada con estereomicroscopio. Se pueden observar las antenas plumosas y la genitalia. Fuente: C. Barceló.

1.2.3.2. Ciclo biológico y comportamiento

Los *Culicoides* tienen un ciclo de desarrollo holometábolo con cuatro fases larvarias (Holbrook 1996) (Fig. 10). Los huevos son depositados en gran variedad de sustratos dependiendo de la especie, en general en zonas cercanas a sus hospedadores y ricas en humedad y materia orgánica (Bravermann *et al*. 1974, Coetzer *et al*. 1994, Zimmer *et al*. 2014a). El número de huevos varía dependiendo de la especie y la temperatura. No son resistentes a la sequía y suelen eclosionar entre los 2 y 7 días (Mellor *et al*. 2000; Meiswinkel *et al*. 2004c).

El primer estadio larvario (L1) mide entre 0.5 y 1 mm (dependiendo de la especie) y emerge del huevo por su parte distal. Algunas especies como *C. sonorensis* tienen un seudópodo en el protórax que les ayuda a salir del huevo (Abubekerov 2014). La larva se desplaza por el substrato con un movimiento serpentiforme típico entre las zonas más superficiales hasta los 12 cm de profundidad (Uslu y Dik 2006). La velocidad de este movimiento aumenta conforme pasa de un estadio al siguiente (Abubekerov 2014). Su aparato bucal esta poco desarrollado con dientes desiguales. Su alimentación es variada y, dependiendo de la especie, va desde materia vegetal hasta protozoos, nematodos, rotíferos y hasta pequeños artrópodos (Mellor *et al*. 2000, Meiswinkel *et al*. 2004c). El periodo de la fase larvaria es muy variable, puede durar entre 4 días y semanas o incluso meses dependiendo de la temperatura y fotoperiodo, de hecho, pueden entrar en diapausa si la temperatura y las horas de luz descienden hasta un determinado umbral (Rawlings y Mellor 1994, Mellor *et al*. 2000). Aparte del tamaño, el segundo (L2) y tercer estadio larvario (L3) se diferencian del primero por un aumento progresivo de la cápsula cefálica y la complejidad de las estructuras de su aparato bucal. El cuarto y último estadio larvario (L4) es el más activo y donde adquiere su tamaño final acumulando grasa como reserva energética para la fase de pupa (González y Goldarazena 2011, Abubekerov 2014).

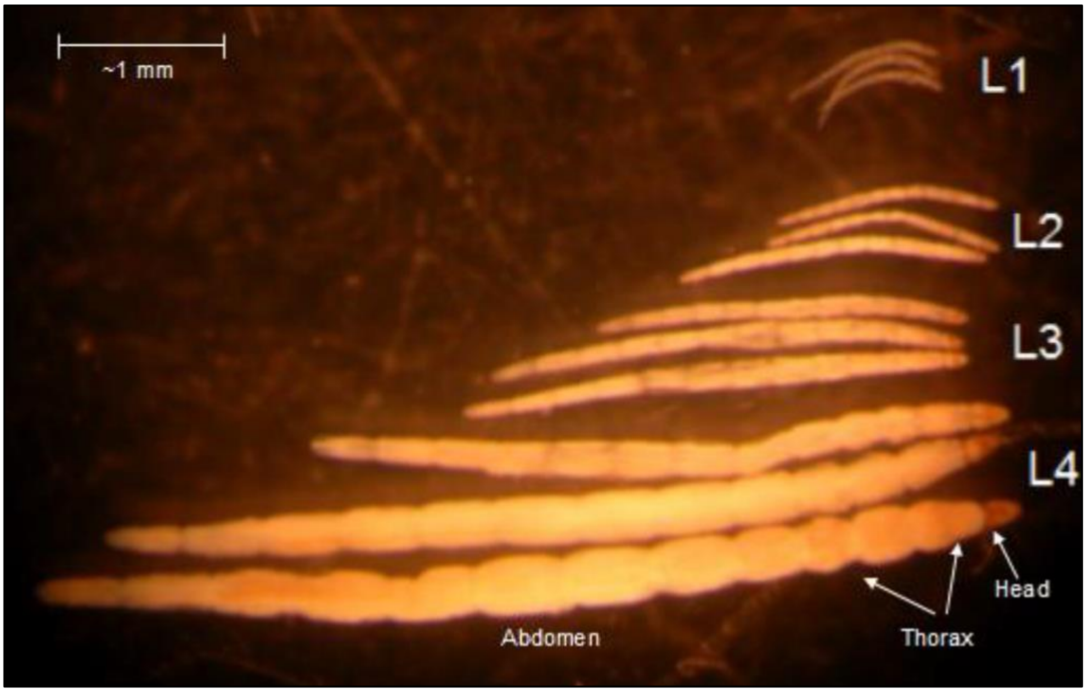


Figura 10. Ejemplo de los 4 estadios larvarios (L1-L4) de *C. sonorensis* donde se observa la diferencia de tamaño y de su cápsula cefálica. Fuente: Abubekerov (2014).

La fase de pupa es la más corta de todas, suele durar entre dos y tres días, llegando excepcionalmente a las cuatro semanas dependiendo de la especie y la temperatura (Mellor *et al*. 2000). Las pupas no se alimentan y suelen encontrarse situadas en la superficie del sustrato para facilitar la emergencia del adulto, aunque tienen la capacidad de moverse hacia zonas inferiores cuando la situación lo requiere (Kettle 1977). Tienen capacidad de flotar en caso de inundación de la zona, a excepción de la especie *Culicoides imicola* Kieffer donde la fase de pupa se interrumpe hasta que el medio esté lo suficientemente seco para el pupado (Nevill 1967).

Los adultos recién emergidos esperan en reposo hasta la esclerotización de su cutícula. Los machos solo se alimentan de néctar de las flores junto con otras substancias azucaradas y suelen emerger antes que las hembras, las cuales pueden empezar a alimentarse de sangre a partir del segundo día de vida (González y Goldarazena 2011). Tienen hábitos crepusculares con picos de actividad durante el amanecer y el anochecer excepto dos especies presentes en Europa que son diurnas: *Culicoides heliophilus* Edwards y *Culicoides dewulfi* Goetghebuer (Mellor *et al*. 2000). La temperatura óptima para la actividad de estos insectos es de entre 13 y 35ºC y pueden estar activos durante todo el año en las regiones más cálidas (Sellers 1980, Verhoef *et al*. 2014).

La hembra pueden alimentarse varias veces del hospedador durante su vida que en general comprende entre los 10 y 20 días (Mellor *et al*. 2000, Purse *et al*. 2015), aunque excepcionalmente pueden llegar a los 90 días en condiciones de laboratorio (Goffredo *et al*. 2004).

Para la cópula, los machos forman un “enjambre nupcial” y atraen a las hembras (Kettle 1995) aunque también se han observado enjambres formados exclusivamente por hembras (Kirkeby 2018). La cópula tiene lugar en pleno vuelo normalmente dentro del mismo enjambre. El macho gira su genitalia 180º para el apareamiento (Fig. 11) aunque todavía se desconocen los detalles de este comportamiento en especies de interés médico-veterinario (Blackwell *et al*. 1992, González *et al*. 2017). La hembra guarda el esperma del macho en una o dos espermatecas que le puede servir para una o más puestas de huevos dependiendo de la especie.

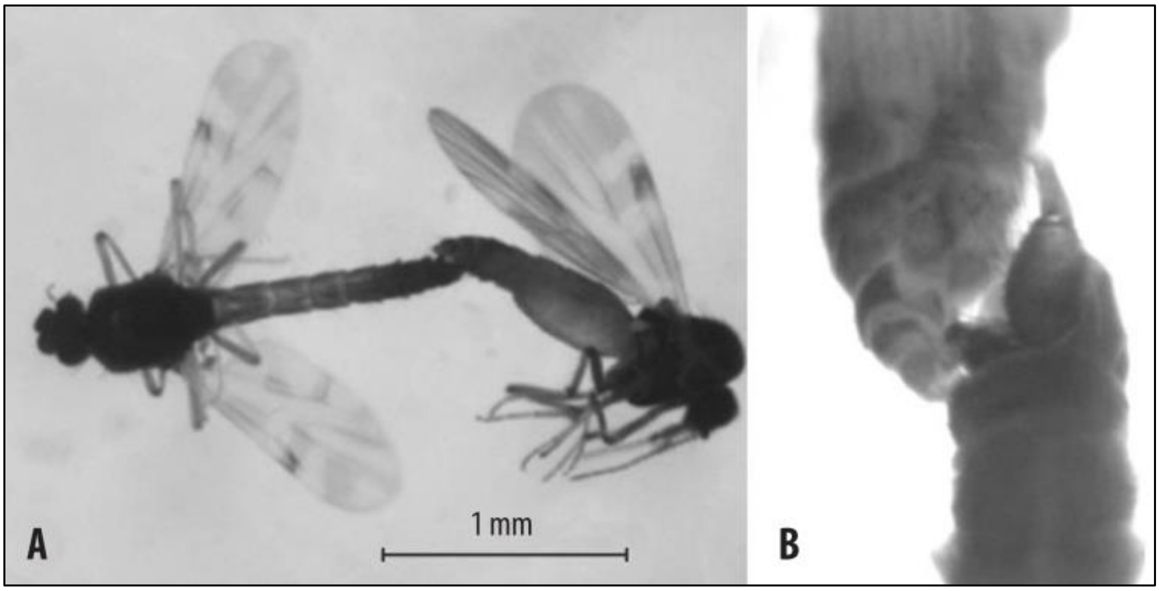


Figura 11. Pareja de *C. obsoletus* apareándose capturada en un enjambre mediante manga entomológica (A) y detalle de la genitalia durante la cópula (B). Fuente: González *et al*. (2017).

Los adultos tienen un rango de dispersión activo corto y no se suelen alejar más de 100 metros desde su punto de cría, donde también se encuentran sus hospedadores, aunque hay casos en los que pueden desplazarse hasta 3 km (Lillie 1981, Mellor *et al*. 2000, Kirkeby *et al*. 2013). No suelen volar los días donde la velocidad del viento supera los 3 m/s (Carpenter *et al*. 2008b, Sanders *et al*. 2011) pero cabe destacar que debido a su pequeño tamaño, pueden ser transportados por el viento, siendo una vía de expansión de *Culicoides* vectores entre distintos países (Sellers *et al*. 1978, Sellers 1980, Alba *et al*. 2004, Hendrickx *et al*. 2008, Jacquet *et al*. 2016).

1.2.3.3. Ciclo gonotrófico y hospedadores

El ciclo de alimentación de las hembras y desarrollo de los huevos que culmina con la puesta se denomina ciclo gonotrófico. Existen cuatro estadios gonotróficos que se pueden diferenciar a simple vista con un estereomicroscopio. Según los trabajos de Dyce (1969), llamamos hembras nulíparas a las hembras sexualmente maduras que todavía no han realizado una oviposición, por lo tanto, corresponde a hembras jóvenes que tienen que alimentarse para obtener la proteína necesaria para la maduración de los huevos y presentan el abdomen transparente (Fig. 12, A). Las hembras grávidas son aquellas que tienen el abdomen lleno de huevos (Fig. 12, B) y las hembras paras (del inglés “parous”) (Fig. 12, C) son las hembras que acaban de ovipositar y todavía tienen el abdomen dilatado, además de pigmentación rojiza en las paredes del abdomen debido a la presencia de restos de sangre del hospedador. Como comentaremos más adelante, las hembras en este estadio gonotrófico son las únicas que presentan capacidad de transmitir arbovirosis. Finalmente, en las hembras alimentadas de sangre (Fig. 12, D) se observa su abdomen dilatado y lleno de la sangre que ha ingerido. Algunos autores como Braverman y Mumcuoglu (2009) y Harrup *et al*. (2013) ponen en duda la validez de este método para diferenciar los estados gonotróficos ya que encontraron hembras nulíparas de *C. imicola* y *C. obsoletus* con pigmentación en el abdomen, sin embargo, sigue siendo la metodología más aceptada para estimar el estado gonotrófico de las hembras de estos insectos (EFSA 2017).

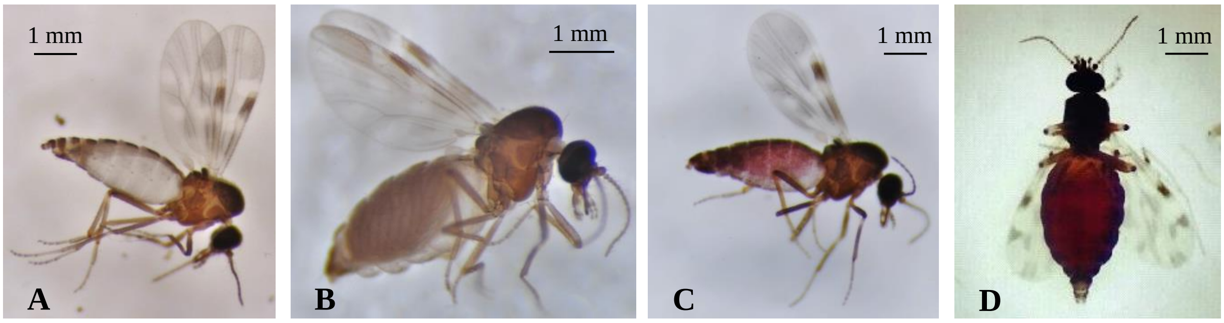


Figura 12. Estados gonotróficos de hembras de *Culicoides* realizadas con estereomicroscopio. Hembra nulípara de *C. obsoletus* (A), grávida de *C. obsoletus* (B), para de *C. obsoletus* (C) y alimentada de sangre de *C. imicola* (D). Fuente: C. Barceló.

1.3. Principales patógenos transmitidos por *Culicoides* spp.

Los dípteros del género *Culicoides* son capaces de transmitir multitud de patógenos. Actualmente se consideran seis familias de arbovirus: Togaviridae, Flaviviridae, Bunyaviridae, Rhabdoviridae, Reoviridae y Orthomyxoviridae (Weaver 2006) de los cuales se han llegado a aislar un total de 75 arbovirus en diferentes especies de *Culicoides*, además de protozoos y nematodos parásitos (Meiswinkel *et al*. 2004c). A continuación se citarán los más representativos:

1.3.1. Familia Reoviridae

1.3.1.1. El virus de la Lengua Azul

El VLA en inglés *Bluetongue* virus (BTV) o también llamado virus de la fiebre catarral ovina es un arbovirus del género *Orbivirus* incluido dentro de la familia Reoviridae que afecta principalmente a rumiantes domésticos y salvajes (Barnard 1997, Verwoerd y Erasmus 2004) causando una elevada mortalidad en el ganado ovino (dependiendo del serotipo), mientras que el ganado bovino suele presentar síntomas subclínicos actuando como reservorio importante de la enfermedad (Taylor 1986, Maclachlan *et al*. 2009, EFSA 2017).

La enfermedad fue descrita por primera vez entre los años 1781 y 1784 por François Levaillant, un biólogo francés que realizó numerosos viajes a Sudáfrica (Hutcheon 1902, Gutsche 1979). En 1943 se demostró que el VLA era transmitido por la picadura de dípteros del género *Culicoides*, concretamente *C. imicola* (Du Toit 1944). Actualmente unas 20 especies de las 1357 existentes se consideran vectores potenciales de la enfermedad (Mellor *et al*. 1990, Coetzer *et al*. 1994) y hoy en día todos los brotes de VLA coinciden con la distribución de especies de *Culicoides* consideradas vectores biológicos de este virus (Tabachnick 2004).

Actualmente existen 27 serotipos diferentes en todo el mundo, los más recientes son el VLA-25 aislado en Suiza (Hofmann *et al*. 2008a), el VLA-26 en Kuwait (Maan *et al*. 2011) y el VLA-27 en Francia (Zientara *et al*. 2014, Schulz *et al*. 2016). En Europa han ido circulando once de ellos, concretamente el 1, 2, 4, 6, 8, 9, 10, 11 y 16 (Zientara y Sánchez-Vizcaíno 2013), de los cuales cinco (1, 2, 4, 8 y 10) han circulado en algún momento por España (Pérez de Diego *et al*. 2014).

Si bien el VLA está ligado a animales domésticos, siendo el ganado ovino el que muestra la sintomatología más severa (Taylor 1986); hay que tener en cuenta también el papel de los animales silvestres, ya que juegan un papel fundamental como reservorio y circulación del virus (Ruiz-Fons *et al*. 2014). En España existen 6 especies que en algún periodo han dado positivo en los test serológicos: el gamo europeo (*D. dama*), el muflón (*Ovis aries musimon*), el ciervo (*C. elaphus*) y el corzo (*C. capreolus*) han dado positivo de los serotipos 1, 4 y 8 mientras que el arruí (*Ammotragus lervia*) y la cabra montesa (*C. pyrenaica*) solo han dado positivo al serotipo 1 (EFSA 2017).

1.3.1.1.1. Ciclo de transmisión de la enfermedad

El ciclo empieza cuando una hembra de *Culicoides* en fase para y con las glándulas salivares infectadas del virus pica a su vertebrado hospedador. En ese momento el virus pasa a su sistema linfático y se reproduce en los nódulos, el endotelio de las arterias, arteriolas y capilares (Gibbs y Greiner 1994). A continuación, el virus llega al torrente sanguíneo donde empieza el periodo de viremia siendo transportado hasta llegar a todos los órganos del cuerpo (MacLachlan 1994). La fase de viremia depende de la vida de las células que transportan el virus, puede durar entre un par de semanas hasta los cien días (Sellers 1980, Bonneau *et al*.

2002, Gubbins *et al*. 2008) y debe alcanzar un nivel mínimo para que la cantidad de virus ingerido sea suficiente para poder ser transmitido por un vector susceptible. Se llama Periodo de Incubación Intrínseco (PII) al tiempo transcurrido entre que el hospedador es infectado hasta que desarrolla la enfermedad (Fig. 13).

Una vez que la sangre infectada es ingerida por el insecto vector, el virus se replica activamente pasando al hemocele y alcanzando otros tejidos del insecto hasta llegar a las glándulas salivares. Es en ese punto cuando el insecto es capaz de transmitir el virus (Mellor *et al*. 2000). El periodo comprendido entre la ingesta de sangre infectada del hospedador hasta la infección de las glándulas salivares del vector se llama Periodo de Incubación Extrínseco (PIE) que puede durar unas dos semanas dependiendo de la temperatura exterior (Mullens *et al*. 2004, Carpenter *et al*. 2011).

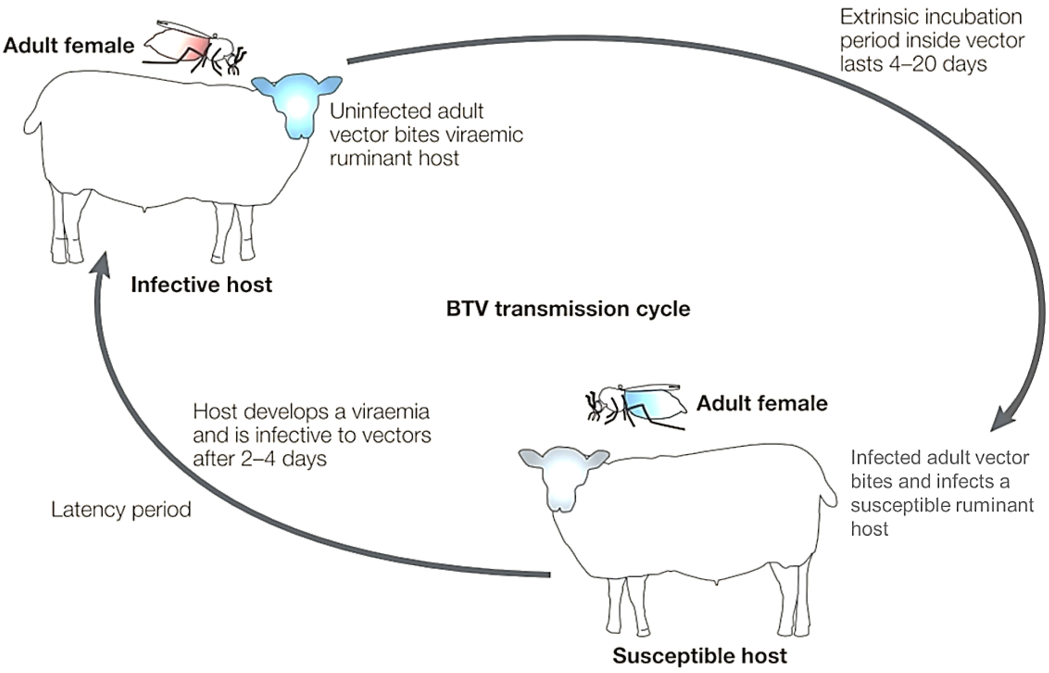


Figura 13. Esquema del ciclo de transmisión del VLA. Fuente: Purse *et al*. (2005)

1.3.1.2. Peste Equina Africana (PEA)

La PEA en inglés *African Horse Sickness* (AHS) es una enfermedad producida por un virus del género *Orbivirus* de la familia Reoviridae igual que el VLA. Se trata de un virus endémico del África Subsahariano que afecta principalmente a caballos (*Equus caballus*) y a otros animales de la familia Equidae (*E. asinus*, *E. caballus* x *asinus*, *Equus zebra*, etc.) (Mellor y Hamblin 2004) causando alteraciones respiratorias y circulatorias con tasa de mortalidad de hasta el 90%. En 1966 hubo el primer brote en España alrededor de Gibraltar y en 1987 fue detectado en un safari de Madrid debido a la importación de cebras infectadas causando graves pérdidas económicas (Rodriguez *et al*. 1992, Sánchez-Vizcaíno 2004). El virus fue erradicado finalmente en 1990 (Sánchez-Matamoros *et al*. 2016). En España, el principal vector es *C. imicola* aunque otras especies como *C. obsoletus* y *C. pulicaris* también pueden estar implicadas en la transmisión de este virus (Mellor *et al*. 1990, Nielsen *et al*. 2010, Meiswinkel *et al*. 2004c).

1.3.1.3. Enfermedad Epizoótica Hemorrágica (EEH)

La EEH en inglés *Epizootic haemorrhagic disease* (EHD) es otra enfermedad producida por un *Orbivirus* muy parecido al VLA que afecta principalmente a diferentes especies de ciervos llegando al 90% de mortalidad aunque el ganado bovino también puede mostrar signos de la enfermedad (Savini *et al*. 2011). En el año 2006 llegó a Marruecos siendo *C. imicola*, *C. obsoletus* y *C. punctatus* los vectores de este virus.

1.3.2. Familia Bunyaviridae

1.3.2.1. Enfermedad de Schmallenberg (SB)

Se trata de una enfermedad de reciente aparición en Europa causada por un virus llamado Schmallenberg (SBV) del género *Orthobunyavirus*. Se detectó por primera vez el año 2011 en ganado vacuno en Alemania (Hoffmann *et al*. 2012) extendiéndose por todo el Norte de Europa y expandiéndose hasta el Mediterráneo posiblemente debido a movimientos de animales infectados y al transporte del vector con el viento (Beer *et al*. 2013, Sedda and Rogers 2013, Afonso *et al*. 2014, Elbers *et al*. 2015). Este virus afecta principalmente al ganado vacuno y ovino aunque los artiodáctilos salvajes como renos y muflones también son portadores del virus (Balseiro *et al*. 2015, García-Bocanegra *et al*. 2017). El primer caso en España fue el año 2012 en Andalucía (EFSA, 2013) considerando el complejo Obsoletus, *C. imicola* y *C. punctatus* los principales vectores del virus en Europa (Larska *et al*. 2013, Veronesi *et al*. 2013, Pagès *et al*. 2017).

1.3.2.2. Virus Akabane (AKAV)

Se trata también de un *Orthobunyavirus* originario de Sudáfrica que afecta a un amplio rango de hospedadores como búfalos (*Syncerus caffer*), ñus (*Connochaetes taurinus*) camellos (*Camelus* spp.), elefantes (*Loxodonta africana*), caballos y rumiantes domésticos (Jennings y Mellor 1989, Meiswinkel *et al*. 2004c). El virus está distribuido por África, Oriente Medio, Sudeste asiático y Australia (Al Busaidy *et al*. 1987, St George and Kirkland 1994, Taylor y

Mellor 1994, Stram *et al*. 2004) y la transmiten especies de vectores diferentes dependiendo de la región geográfica, entre ellos *C. imicola* (Blackburn *et al*. 1985, Al Busaidy y Mellor 1991), además, algunas especies de culícidos del género *Aedes* y *Culex* también se consideran capaces de transmitir el virus (Oya *et al*. 1961, Metselaar y Robin 1976).

1.3.3. Protozoos parásitos

Los hemosporidios son un orden de protozoos parásitos dentro del filo Apicomplexa que se transmiten por picaduras de artrópodos. Los dípteros del género *Culicoides* (e.g. *C. impunctatus*) son capaces de transmitir especies del género *Plasmodium*, *Haemoproteus* y *Leucocytozoon* que suelen parasitar principalmente a aves (Morimoto *et al*. 2015, Žiegytė *et al*. 2017). En España están presentes las especies *C. circumscriptus* y *C. paolae* que además de presentar hábitos ornitofílicas, también puede alimentarse de mamíferos y el hombre, por lo tanto, podrían llegar a actuar como vectores de estos patógenos (Ferraguti *et al*. 2013, Veiga *et al*. 2018).

1.5. Especies implicadas en la transmisión del VLA en España

1.5.1. Subgénero *Avaritia* Fox, 1955

1.5.1.1. Complejo Imicola

Se trata de un complejo formado por doce especies, tres de ellas consideradas vectores importantes del VLA: *Culicoides brevitarsis* Kieffer, *Culicoides bolitinos* Meiswinkel y *C. imicola* (Meiswinkel *et al*. 2004c), siendo este último el único presente en España y Europa con un patrón de manchas alares inconfundible (Fig. 13, A).

Culicoides imicola Kieffer, 1913

Se trata de una de las especies más importantes implicadas en la transmisión del VLA en Europa y una de las más abundantes junto con las especies del complejo Obsoletus (Braverman *et al*. 1985, Mellor y Wittmann 2002, Purse *et al*. 2007, Calvete *et al*. 2009a, Wilson y Mellor 2009). Ocupa la franja sur de Europa, concretamente se encuentra presente por lo menos en siete países: Chipre, Malta, Grecia, Italia, Francia, Portugal y España (Versteirt *et al*. 2017). Durante los últimos años, *C. imicola* ha ido aumentando su rango de distribución debido a los efectos del cambio climático (Purse *et al*. 2005, Calvo *et al*. 2009, Samy y Peterson 2016) y se registró una captura puntual en Suiza siendo la zona más al norte donde ha llegado anecdóticamente esta especie (Cagienard *et al*. 2006). En España se encuentra localizado en las regiones del suroeste de la Península Ibérica y el archipiélago

Balear (Ortega *et al*. 1998, Miranda *et al*. 2003, Calvete *et al*. 2008, Calvete *et al*. 2009b, Acevedo *et al*. 2010, Talavera *et al*. 2015, Cuéllar *et al*. 2018b).

Sus larvas crían en zonas no inundadas y ricas en materia orgánica (Capela *et al*. 1993, Foxi y Delrio 2010).Las hembras alimentadas de sangre pueden servir para determinar los hospedadores de los que se alimentan gracias a técnicas moleculares (Lassen *et al*. 2012, Martínez-de la Puente *et al*. 2012, 2015, 2017), además algunos autores aseguran que los hábitos alimentarios se pueden identificar gracias a determinados caracteres morfológicos de las antenas y palpos maxilares (Augot *et al*. 2017a). De hecho, las hembras adultas de *C. imicola* son generalmente mamófilas y se alimentan principalmente de sangre de bóvidos (*Ovis aries*, *Capra hircus*, *Cervus elaphus* y *Bos taurus*) pero también pueden alimentarse de sangre de caballos (*Equus caballus*), humanos (*Homo sapiens*), perros (*Canis lupus familiaris*), cerdos (*Sus scrofa domestica*) (Martínez-de la Puente *et al*. 2017, Talavera *et al*. 2017b) e incluso aves como *Lanius meridionalis* (Braverman y Phelps 1981, Slama *et al*. 2015).

1.5.1.2. Complejo Obsoletus

Se trata del grupo de especies más comunes en el centro y norte de Europa (Rawlings y Mellor 1994, Hoffmann *et al*. 2008, Meiswinkel *et al*. 2008, Cuéllar *et al*. 2018b). En España se encuentran distribuidas en toda la península Ibérica pero con mayor abundancia en la mitad norte y Baleares (Miranda *et al*. 2003, Calvete *et al*. 2008, Lucientes *et al*. 2008, Calvete *et al*. 2009b, Ducheyne *et al*. 2013, Talavera *et al*. 2015).

Actualmente se consideran cinco especies en este complejo que se encuentran en toda Europa pero con distinta distribución dependiendo de la región en la que nos encontremos. *Culicoides scoticus* Downes and Kettle y *C. obsoletus* son las más representativas en España y centro de Europa (Sarto I Monteys y Saiz-Ardanaz 2003, Pagès y Sarto I Monteys 2005, Hofmann *et al*. 2008b, Alarcón-Elbal *et al*. 2016). *Culicoides chiopterus* (Meigen) y *C. dewulfi* suelen estar restringidas a zonas más al centro y norte de Europa pero también se pueden encontrar en España (Alarcón-Elbal *et al*. 2016, Kluiters y Pagès 2016). Finalmente, *Culicoides montanus* (Shakirzjanova), a pesar de que su estatus taxonómico está actualmente cuestionándose (Mingotte *et al*. 2018), es una especie menos abundante y está distribuida principalmente en el sur de Italia, Sicilia, Cerdeña (Monaco *et al*. 2010), también en Portugal (Ribeiro *et al*. 2015) y recientemente en las Islas Baleares (Tabourin *et al*. 2017). Cabe destacar que también se ha descrito una especie críptica llamada "dark *obsoletus*" en Países Bajos (Meiswinkel *et al*. 2015).

Las hembras del complejo Obsoletus no se pueden determinar mediante morfología con estereomicroscopio ya que tienen un patrón de manchas alares idéntico o muy parecido (Fig. 13, B) y es necesario montar preparaciones microscópicas de las genitalias (Nielsen y Kristensen 2011) y/o técnicas moleculares para su identificación a nivel de especie (Ander *et al*. 2013, Garros *et al*. 2014, Harrup *et al*. 2015).

Presentan una gran diversidad de puntos de cría tales como el estiércol fresco, residuos de ensilaje, estiércol adherido a las paredes, materia orgánica de los gallineros, pilas de compost, madera en descomposición, etc. (Kettle y Lawson 1952, Mellor y Pitzolis 1979, Harrup *et al*. 2013, Zimmer *et al*. 2013a, 2014b, Steinke *et al*. 2016, Braverman *et al*. 2018).

Al igual que *C. imicola*, las hembras son mamófilas y con una amplia gama de hospedadores. Se alimentan principalmente de sangre de rumiantes domésticos (*O. aries*, *C. hircus* y *B. taurus*) (Martínez-de la Puente *et al*. 2012, Pettersson *et al*. 2013) además de ciervos, corzos, gamos y cabras montesas (*C. elaphus*, *Capreolus capreolus*, *Dama dama* y *Capra pyrenaica*), caballos, cerdos, conejos (*Oryctolagus cuniculus*), ratones (*Mus musculus*), humanos e incluso aves como la paloma torcaz (*Columba palumbus*) o el ánade real (*Anas platyrhynchos*) (Martínez-de la Puente *et al*. 2015, Talavera *et al*. 2017b).

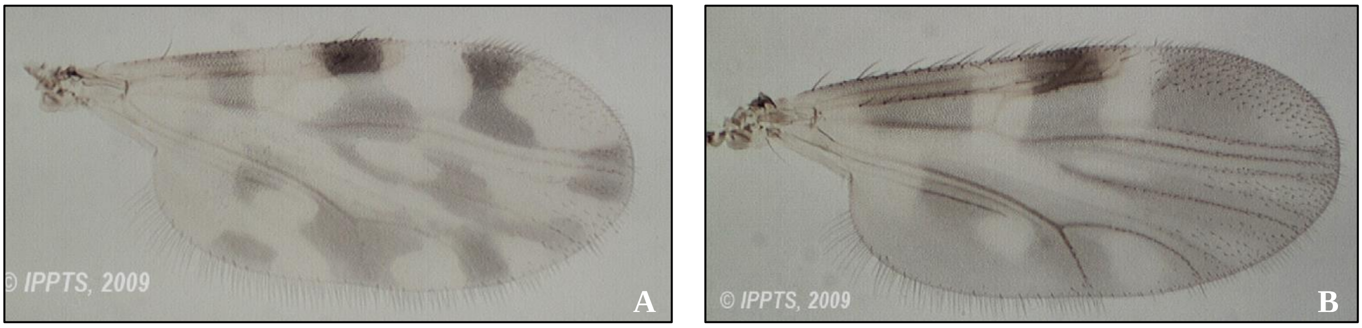


Figura 14. Patrón de manchas alares de *C. imicola* (A) y *C. obsoletus* (B). Fuente: Mathieu *et al*. (2012).

1.5.2. Subgénero *Culicoides* Latreille, 1809

1.5.2.1. Complejo Pulicaris

Actualmente este complejo de especies incluye: *Culicoides pulicaris* (L.), *Culicoides lupicaris* Downes & Kettle, *C. impunctatus*, *Culicoides punctatus* Meigen, *Culicoides newsteadi* Austen, *Culicoides grisescens* Edwards, *Culicoides magnus* Colaço y *Culicoides* *bysta* n. sp. (Meiswinkel *et al*. 2004a, Nolan *et al*. 2007, Sarvašová *et al*. 2017). Estos tres últimos no se encuentran en España.

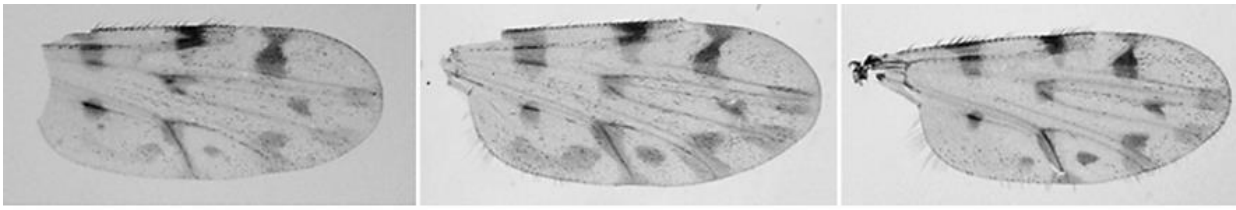
Algunos autores consideran *C. newsteadi* dentro de un complejo aparte llamado Newsteadi donde se incluyen tres especies crípticas de *C. newsteadi* con patrón de manchas alares muy parecido (Fig. 14) (Pagès *et al*. 2009). Por otro lado, se han descrito especies crípticas de *C. pulicaris* en Turquía (Yildirim *et al*. 2018) y en España (Foxi *et al*. 2016) como *Culicoides cryptipulicaris* sp. nov. y *Culicoides quasipulicaris* sp. nov. (Talavera *et al*. 2017a).

Las especies *C. pulicaris*, *C. lupicaris*, *C. punctatus* y *C. newsteadi* son actualmente las únicas implicadas en la transmisión del VLA (Mellor *et al*. 1981, Caracappa *et al*. 2003, Ferrari *et al*. 2005, Goffredo *et al*. 2015, Foxi *et al*. 2016).

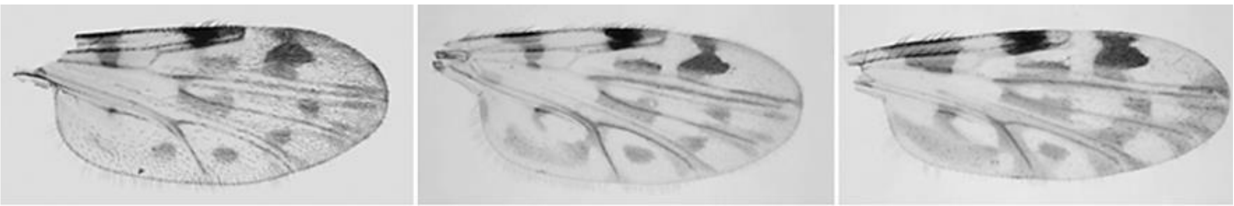
Los más abundantes en España son *C. pulicaris* seguido de *C. newsteadi*. El primero es más abundante en la mitad norte de la península Ibérica incluyendo las Islas Baleares siguiendo una distribución similar a las especies del complejo Obsoletus (Miranda *et al*. 2004, Lucientes *et al*. 2008, Ducheyne *et al*. 2013, Cuéllar *et al*. 2018b) mientras que *C. newsteadi* se encuentra en zonas más al interior y sur de la península y Baleares (Ortega *et al*. 1999, Del Río *et al*. 2013, Ducheyne *et al*. 2013).

Las larvas de *C. pulicaris* crían en pastos abiertos, zonas fangosas y pantanos (Kettle y Lawson 1952, Campbell y Pelham-Clinton 1960, Harrup *et al*. 2013) y *C. newsteadi* en márgenes de estanques con vegetación asociados a animales de granja (Foxi y Delrio 2010).

Las hembras adultas de *C. pulicaris* se alimentan de rumiantes (*O. aries*, *C. hircus*, *B. taurus*, *C. elaphus* y *D. dama*), caballos, humanos y gallinas (*Gallus gallus*) (Pettersson *et al*. 2013, Martínez-de la Puente *et al*. 2015, Talavera *et al*. 2017b). Por otra parte, *C. newsteadi* parece ser estrictamente mamófilo ya que muestra preferencia por vacas, ovejas, ponis y humanos (Elbers y Meiswinkel 2015, Martínez-de la Puente *et al*. 2015).



(a) *C. pulicaris* P1 (b) *C. lupicaris* (c) *C. pulicaris* P3



(g) *C. newsteadi* N1 (h) *C. newsteadi* N2 (i) *C. newsteadi* N3

Figura 15. Detalle del patrón de manchas alares de dos especies del complejo Pulicaris presentes en España donde también se comparan las dos especies crípticas de *C. pulicaris* (arriba). Detalle del patrón de manchas alares de las tres especies crípticas del complejo Newsteadi (abajo). Fuente: Pagès (2009).

1.6. Origen y distribución de la enfermedad en Europa y España

El VLA entró por primera vez en Europa por la cuenca Mediterránea, concretamente los serotipos 4 y 16 en la isla de Chipre el año 1943, siendo la primera vez que el virus se detectaba fuera de África (Gambles 1949).

El primer brote de VLA en España fue en 1956 providente de Portugal, concretamente el serotipo 10 causando la muerte de aproximadamente 180,000 animales (Calvete *et al*. 2009b, Pérez de Diego *et al*. 2014). El brote fue finalmente erradicado en 1958 gracias a restricción de movimientos de animales, campañas de vacunación y el sacrificio de animales infectados.

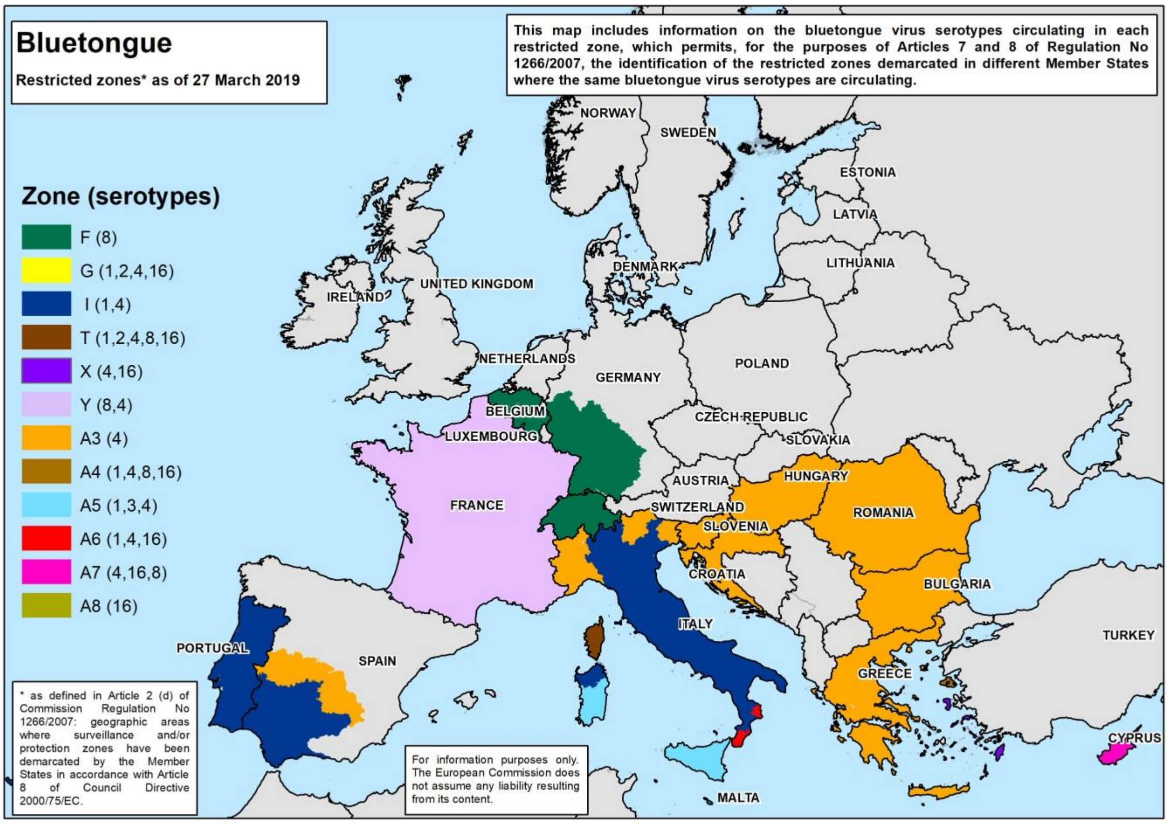
Hasta 1998 en Europa solo hubo un pequeño brote de VLA en Grecia entre 1979 y 1980 (Mertens *et al*. 2008). En España volvió a aparecer en septiembre de 2000 en las islas de Mallorca y Menorca (Islas Baleares), esta vez fue el serotipo 2 probablemente debido al transporte de vectores por corrientes de aire desde Cerdeña y/o desde el Norte de África (Alba *et al*. 2004, Calistri *et al*. 2004, Gómez-Tejedor 2004) causando la muerte de unos 10,000 animales incluyendo los individuos sacrificados (Martínez *et al*. 2005). En 2002 el brote fue erradicado completamente considerando las especies *C. imicola* y *C. obsoletus* como principales vectores ya que estaban presentes durante el brote de la enfermedad (Miranda *et al*. 2003).

Un año después, en 2003, apareció un nuevo caso de LA otra vez en las Islas Baleares que solo afectó a la isla de Menorca, en esta ocasión el brote fue debido al serotipo 4 que fue finalmente erradicado en 2004 (Breard *et al.* 2007, Wilson y Mellor 2009). Ese mismo serotipo (VLA-4) llegó a la península Ibérica, concretamente en Cádiz, debido probablemente al transporte pasivo de vectores desde Marruecos. El virus se extendió hacia el norte llegando a la provincia de Castilla y León. Se vacunaron más de 10 millones de ovejas con vacunas inactivadas. La mortalidad en este brote fue muy baja (Pérez de Diego *et al.* 2014).

En 2007 otro serotipo se detectó en Tarifa (Cádiz), esta vez era el serotipo 1 mucho más virulento que el VLA-4 causando una mortalidad del 7% en animales domésticos y afectando incluso a los rumiantes silvestres como el muflón (Fernández-Pacheco *et al.* 2008). Su llegada se asocia una vez más a la llegada de vectores con el viento (Pérez de Diego *et al.* 2014).

En el 2008 llega un nuevo serotipo al norte de España en Cantabria, el VLA-8, providente del Norte de Europa, concretamente de Bélgica donde llevaba circulando desde el 2006 (Toussaint *et al.* 2007). En esta ocasión, la expansión del virus fue debido al movimiento de animales infectados (De Koeijer *et al.* 2011). Este serotipo causa sintomatología clínica tanto en ganado ovino como vacuno con diferentes grados de severidad. La especie asociada como vector en este caso fue *C. obsoletus* debido a que es la especie predominante en el norte de España donde se dieron los brotes de este serotipo. En 2013 España se declaró libre de este serotipo quedando restringido en Francia.

En la actualidad, cinco serotipos siguen circulando por Europa (Fig. 16). El VLA-1 y VLA-4 siguen presentes en la Península Ibérica. La mayoría de los casos de VLA-1 están concentrados en Portugal, Andalucía y Extremadura mientras que el VLA-4 está más extendido por el Norte hasta Castilla La Mancha.



This map includes information on the bluetongue virus serotypes circulating in each restricted zone, which permits, for the purposes of Articles 7 and 8 of Regulation No 1266/2007, the identification of the restricted zones demarcated in different Member States where the same bluetongue virus serotypes are circulating.

* as defined in Article 2 (d) of Commission Regulation No 1266/2007: geographic areas where surveillance and/or protection zones have been demarcated by the Member States in accordance with Article 8 of Council Directive 2000/75/EC.

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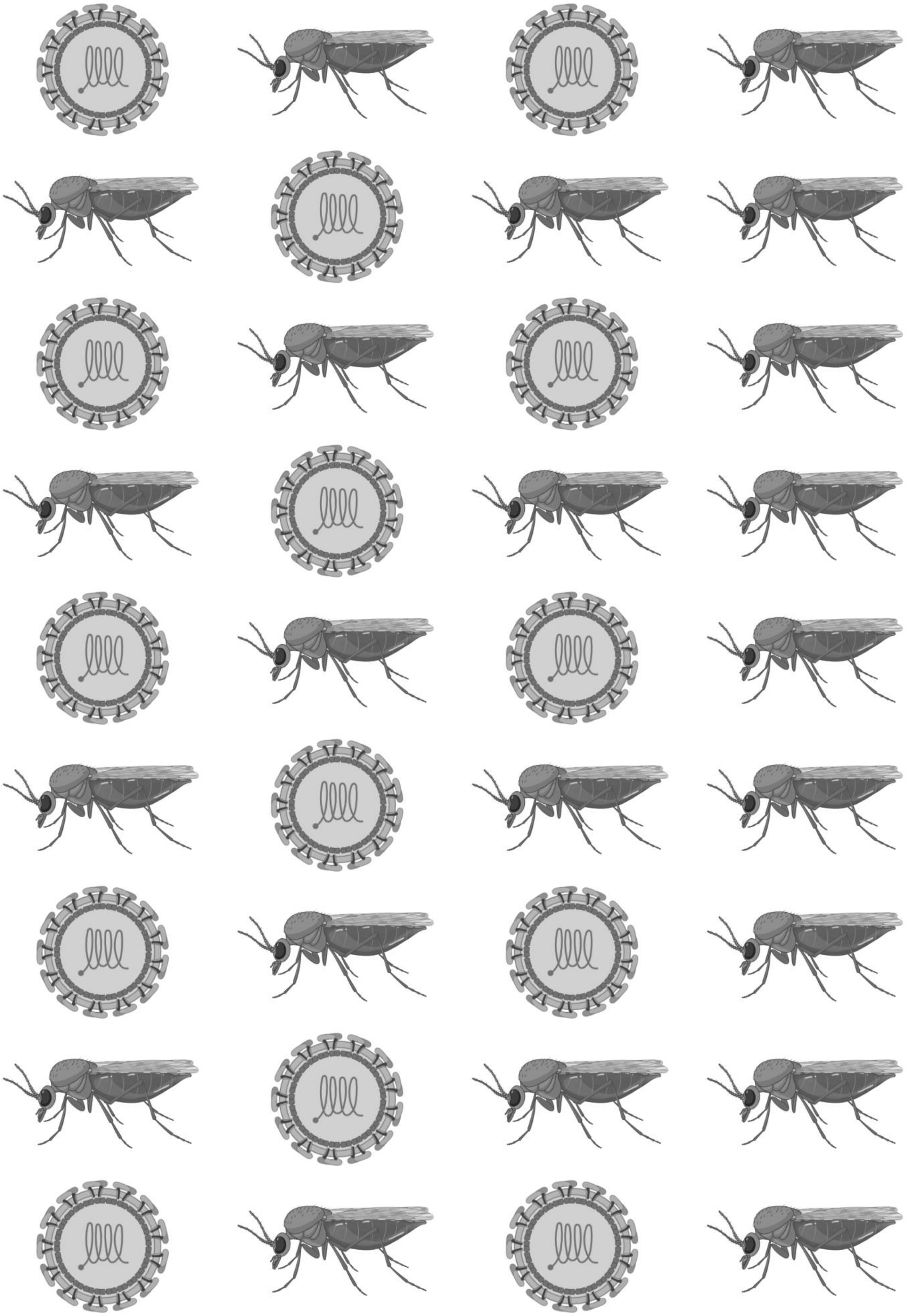
Figura 16. Mapa de las zonas de restricción de animales debido al VLA en Europa (actualizado a marzo de 2019) donde se representan los diferentes serotipos circulando en los países y regiones europeas. Fuente: Comisión Europea (2019)

2. Objetivos del estudio

El presente trabajo tiene dos objetivos fundamentales:

1. Estudiar la dinámica poblacional y fenología de especies de *Culicoides* vectores del VLA en España, donde:
    - Se analizará la distribución y estacionalidad de hembras paras y nulíparas.
    - Se determinarán los factores ambientales implicados en la fenología de estas especies gracias a Modelos Bayesianos Generalizados Lineales Mixtos (GLMM).
    - Se estudiarán los factores ambientales incluyendo el efecto indirecto de la abundancia poblacional de *Culicoides* sobre el periodo de actividad de hembras nulíparas y paras gracias a los Análisis de Trayectorias (*Path analysis models*).
2. Estudiar la bionomía de *Culicoides* asociados a granjas en las Islas Baleares, donde:
    - Se determinarán los valores bionómicos básicos de diferentes especies en condiciones de laboratorio comparando distintos métodos con la finalidad de establecer una futura colonia en cautividad.
    - Se compararán resultados bionómicos de individuos a distintas temperaturas utilizando dos poblaciones diferentes.

3. A Mondrian matrix of seasonal patterns of *Culicoides* nulliparous and parous females at different latitudes in Spain.



3. A Mondrian matrix of seasonal patterns of *Culicoides* nulliparous and parous females at different latitudes in Spain.

3.1. Introduction

The vector seasonality of *Culicoides* species mostly depends on climate, which modulates the feeding, development, survival rates, reproductive activity and fecundity (Purse *et al*. 2015). The seasonal pattern of these insects has been described as uni-, bi-, tri- or multivoltine (Braverman *et al*. 1985), and it is widely accepted that the seasonal transmission of BTV coincides with the period of higher adult abundance of the competent vector species (Mellor *et al*. 2000, Capela *et al*. 2003, Miranda *et al*. 2004, De Liberato *et al*. 2010). The annual and seasonal fluctuation of adult abundance is measured by National programs for the surveillance of the vector species *C. imicola* and the vector species included in the Obsoletus and Pulicaris groups implemented in all the BTV affected European countries (reviewed by Versteirt *et al*. 2017). Published data, mainly focused on spatial distribution, is available in many countries summarized in Table 1.

Table 1. List of countries with National surveillance programs for *Culicoides* vector species in Europe with references.

Country	References
Spain	(Ortega *et al*. 1997, 1998, 1999, Rawlings *et al*. 1998, Sarto I Monteys and Saiz-Ardanaz 2003, Miranda *et al*. 2003, 2004, Calvete *et al*. 2006, 2008, 2009b, Romón *et al*. 2012, González *et al*. 2013a)
Portugal	(Capela *et al*. 1993, 2003, Rawlings *et al*. 1998, Ribeiro *et al*. 2015, Ramilo *et al*. 2017)
France	(Baldet *et al*. 2004, Venail *et al*. 2012, Balenghien *et al*. 2014)
Italy	(Calistri *et al*. 2003, Conte *et al*. 2003, De Liberato *et al*. 2010, Foxi and Delrio 2010, Pili *et al*. 2010, Foxi *et al*. 2016)
Greece	(Patakakis 2004)
The Netherlands	(Meiswinkel *et al*. 2008, Takken *et al*. 2008)
Belgium	(De Deken *et al*. 2008, Fassotte *et al*. 2008, De Regge *et al*. 2015, Sohier *et al*. 2018)
Sweden	(Nielsen *et al*. 2010)
Austria	(Brugger and Rubel 2013, Brugger *et al*. 2016)
Czech Republic	(Rádrová *et al*. 2015)

Table 1. Continued

Germany	(Hoffmann *et al*. 2009)
Great Britain	(Sanders *et al*. 2011)
Romania	(Oprescu *et al*. 2008)
Slovakia	(Sarvašová *et al*. 2016)
Switzerland	(Kaufmann *et al*. 2012)

The *Culicoides* female gonotrophic stage is crucial in BTV transmission. Transmission requires that the adult female takes an infected blood meal, replicates the virus to transmissible levels during the Extrinsic Incubation Period (EIP), and then once this period has been completed, survives to take a subsequent blood meal in which she infects a naive host. The surveillance of *Culicoides* females carried out with light trap samplings often distinguishes between the gonotrophic stage of nulliparous (NF) (newly emerged unpigmented adult females) and parous adult females (PF) that have become pigmented according to Dyce (1969) either as consequence of taking blood meal or aging through the first gonotrophic cycle (Braverman *et al*. 1985). Overall PF are the proportion of the population that may carry transmissible infections whilst NF will not. Therefore, the first detection and seasonal occurrence of *Culicoides* PF have a great importance for the estimation of risk of transmission of BTV. This risk will depend on the presence or absence of this group of females for certain vector species, in addition to the temperature and the determination of its Seasonally Vector-free Period (SVFP) (EFSA 2008). Studies of *C. sonorensis* conducted in California (USA) and Canada (Lysyk 2007, Mayo *et al*. 2014) and others with *C. imicola* in South Africa (Venter *et al*. 1997) and *C. obsoletus* in Russia (Glukhova and Brodskaya 1994) included the parity of in their results. However, the activity of the PF population has been poorly explored for all BTV affected countries in Europe or even Australia (Melville *et al*. 2015) or North America (Ruder *et al*. 2015). Recent surveillance studies such as Rádrová *et al*. (2015) or Foxi *et al*. (2016) are the only ones in Europe that have distinguished PF from NF seasonality in their results.

European Regulation (EC) 1266/20072 for the control, monitoring, surveillance and restrictions on movements of certain animals of susceptible species in relation to BT, define the SVFP as the complete absence of adult *C. imicola* and less than five PF captured in light traps for the other *Culicoides* vector species. Almost all of the European monitoring programs for BTV include the age grading of females and estimate the varying proportions of non-parous and PF through the year. In fact, some studies have used *Culicoides* parous rates as estimator of biting and oviposition survival rates (Birley and Boorman 1982, Holmes and Boorman 1987). Although the method of Dyce (1969) has some limitations (Braverman and Mumcuoglu 2009), it is currently the most used and accepted methodology for estimating the gonotrophic status of females. Hence, in order to provide a deeper knowledge of the seasonal *Culicoides* activity in Spain, we decided to analyse data of the seasonal pattern of the four vector species of *Culicoides* present in Spain: Obsoletus complex and *C. pulicaris* / *C. imicola* and *C. newsteadi* across a North-South axis in the Iberian Peninsula considering how patterns vary between NF and PF; and also for understanding the BTV transmission determining the SVFP in the country.

3.2. Material and Methods

An analysis of the seasonal data, namely maximum monthly abundance of NF and PF in light traps of Obsoletus complex, *C. pulicaris*, *C. imicola*, and *C. newsteadi* species from 2008 to 2010 was carried out across 329 weekly sampling stations. These stations were located in 50 provinces in Spain during the National Entomosurveillance Program for BTV (sponsored by the Spanish Ministry of Rural and Marine Environment).

In addition, to quantify the seasonal differences among latitudes, seven provinces were selected according to their position from North to South axis: Navarra (42º 49’ 00’’ N; 1º 39’ 00’’ O), Zaragoza (41º 35’ 00’’ N; 1º 00’ 00’’ O), Guadalajara (40º 50’ 00’’ N; 2º 30’ 00’’ O), Toledo (39º 50’ 00’’ N; 4º 00’ 00’’ O), Ciudad Real (39º 00’ 00’’ N; 4º 00’ 00’’ O), Córdoba (38º 00’ 00’’ N; 4º 50’ 00’’ O) and Cádiz (36º 30’ 00’’ N; 5º 45’ 00’’ O). These provinces included 49 sampling stations across 46 locations (Fig. 17). The stations were trapped permanently during that period, on a weekly basis. Each sampling station consisted in a CDC-Miniature trap (John W. Hock® Cp., Gainesville, FL, U.S.A.) with a 4-W UV light and photoelectric cell fitted to a suction fan and connected to main power and a collector filled with ethanol and ethylene glycol in water. The traps were set at 1.7-2.0 metres from the floor and placed between 1 and 30 metres from livestock from dusk till dawn. The farms were located between 200 and 797 meters above the sea level and housed between 16 and 3000 large livestock including horses, sheep and cattle.

The maximum number of individuals collected weekly per trap night was depicted in a single Mondrian matrix which consists of a matrix with squares colour-coded according to the abundances of NF and PF of each *Culicoides* species in each month (Meiswinkel *et al*. 2014). The SPSS Statistics® 17.0 software was used to analyse the data. Analyses were subjected to non-parametric tests: one-way Kruskal-Wallis analysis of variance (K-W) for comparing *Culicoides* abundance among seasons and the two-sample Kolmogórov-Smirnov (K-S) in order to assess the differences in abundance across seasons for each species in Spain.

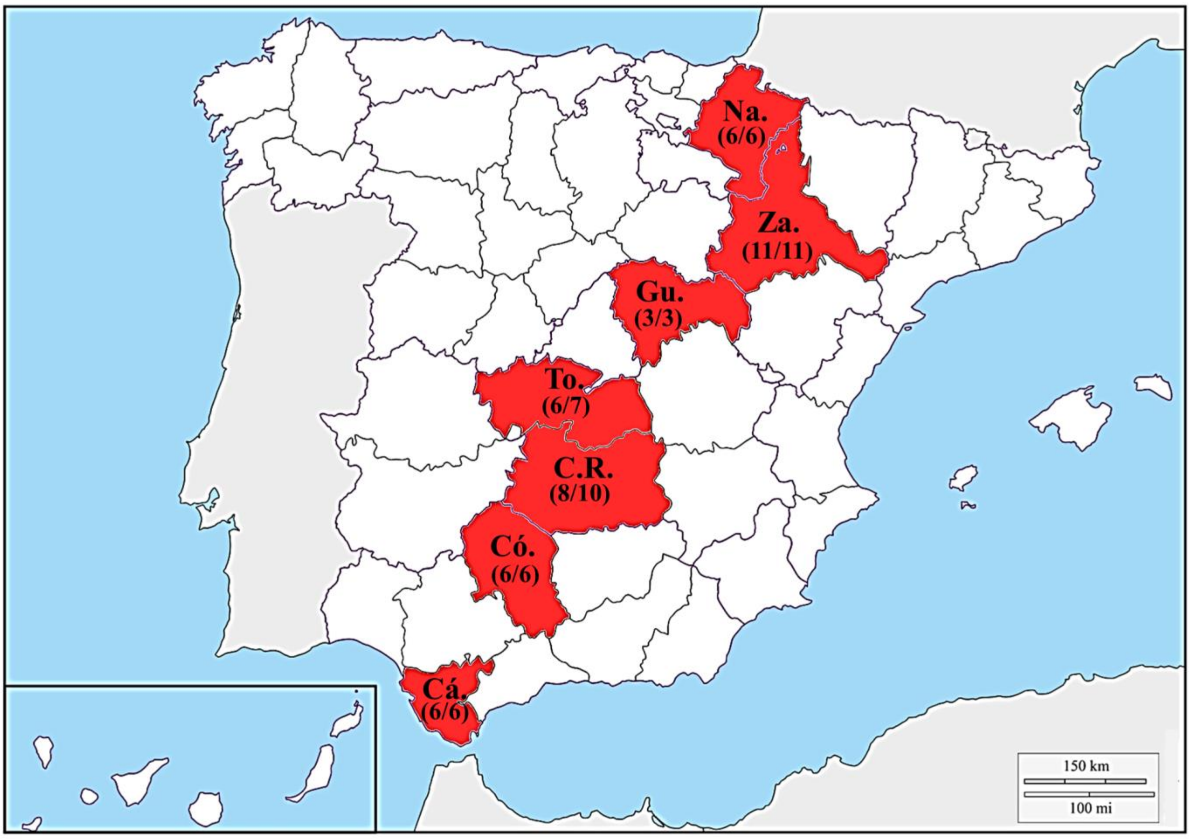


Figure 17. Location of the provinces included in the analysis of maximum monthly catches of nulliparous females (NF) and parous females (PF) of Obsoletus complex, *C. pulicaris*, *C. imicola* and *C. newsteadi* from North to South of mainland Spain. Navarra (Na.), Zaragoza (Za.), Guadalajara (Gu.), Toledo (To), Ciudad Real (C.R.), Córdoba (Có.) and Cádiz (Cá.). In brackets: number of selected locations / number sampling stations.

3.3. Results

3.3.1. General seasonal pattern and abundance of *Culicoides* NF and PF in Spain between 2008 and 2010.

The highest percentage of the annual total of PF for all *Culicoides* species was obtained in summer (between 57.4% and 47.3%, Table 2). Conversely, the proportion of the annual total of NF for all *Culicoides* was highest during spring, with the exception of *C. imicola* that had a higher percentage of the annual of total NF in summer suggesting that adult emergence is still continuing strongly in this species in autumn. As expected, winter was the season with the lowest percentage of the annual total of NF and PF.

Regarding the general weekly seasonal pattern of abundance of NF and PF, results are exhibited as a Mondrian matrix in Figure 18 for each species included in this work:

Obsoletus complex: Individuals of this species were detected through the whole year. Abundances of NF and PF were significantly greater than the abundance of the other three species during winter and spring months (2-tailed K-S tests, $P<0.05$) (Fig. 18). In addition, Obsoletus complex showed higher significant abundance than NF and PF of both *C. newsteadi* and *C. pulicaris* during summer and autumn seasons (2-tailed K-S tests, $P<0.05$), with the exception of *C. newsteadi* PF in autumn.

The first peak of abundance of Obsoletus complex PF was located between April and May and a second peak in June-July. The year 2008 was the one with higher number of captures along the year; being 3972 the highest number of PF collected in April 2008 (Fig. 18). In fact, the number of PF was above the 500 *Culicoides*/trap/night (*C*/t/n) during 5 months from April to August 2008 and over 200 *C*/t/n from March to October 2008 (8 months). December 2008 showed the lowest PF captures (57 *C*/t/n). Only the last two weeks of 2008 were free of PF of this species during that year.

The years 2009 and 2010 showed low abundance of Obsoletus complex. In fact, 2010 was the year with the lowest abundance of Obsoletus complex species with no more than the 399 PF *C*/t/n recorded in early August.

Culicoides pulicaris: We found also a bimodal pattern of seasonal abundance of PF for this species with a first peak recorded between April and May and a second peak located between June and July. The number of collected individuals was lower compared to Obsoletus complex species. The maximum number of individuals was 308 *C*/t/n collected in June 2008

(Fig. 18). As well as Obsoletus complex, the 2008 was the year with the highest abundance of *C. pulicaris*, with PF reaching weekly maximum values of between 10-100 *C*/t/n from April to September. No single specimen was collected during January and December 2008, with the exception of one single NF individual collected on January.

Similarly to Obsoletus complex, *C. pulicaris* abundance during 2009 and 2010 was lower than 2008 with long periods free of PF of this species. In fact, this species showed the longest free-period in Spain: from early November 2008 to mid-May 2009 (more than six months). In 2010, the species showed six non-consecutive months of vector-free period, from January to mid-March and from early September to December.

Culicoides imicola: Different from Obsoletus complex and *C. pulicaris*, the highest percentage of the annual total of maximum catches of both NF and PF was recorded in summer (Table 2). In addition, *C. imicola* showed higher absolute abundance of NF and PF than *C. newsteadi* and *C. pulicaris* during summer and autumn seasons (2-tailed K-S tests, $P<0.05$) (Fig. 18); with the exception of *C. pulicaris* PF in summer. The number of captures of PF tripled in a month during the peaks detected from July to August and from October to November. In that case, the abundance of this species was highest in 2010 with six-seven months above the 500 PF *C*/t/n (from late May to November).

The abundance of *C. imicola* PF was lowest in 2009 with no more than 511 individuals recorded in mid-September. In fact, the longest free-period for this species was from December 2008 to mid-April 2009 (more than four months).

Culicoides newsteadi: The abundance of this species was lower than that of *C. imicola* and the Obsoletus complex. However, this species showed significant higher abundance of PF than *C. pulicaris* during summer and autumn seasons (2-tailed K-S tests, $P<0.05$) (Fig. 18). The peak of PF was detected between May and July with maximum captures of 556 individuals recorded on June 2010. The abundance of this species was highest in 2008.

Conversely, PF were absent from December to January across all three years. The abundance of this species was lowest in 2009, in which *C. newsteadi* was absent for more than five months: from November 2008 to late April 2009.

Table 2. Percentage of the annual total maximum catches of nulliparous females (NF) and parous females (PF) for each *Culicoides* species that were captured in each season during the years 2008 and 2010 in mainland Spain.

	Total *Culicoides*		***C. imicola***		**Obsoletus complex**		***C. newsteadi***		***C. pulicaris***	
Season	**NF**	**PF**	**NF**	**PF**	**NF**	**PF**	**NF**	**PF**	**NF**	**PF**
Spring	37.1%	27.1%	3.8 %	6.0 %	45.2 %	35.5 %	43.9 %	27.5 %	52.1 %	33.7 %
Summer	40.3%	50.5%	48.5 %	54.7 %	38.0 %	47.3 %	37.9 %	52.2 %	42.8 %	57.4 %
Autumn	15.7%	18.8%	46.9 %	38.6 %	7.8 %	11.8 %	12.1 %	18.1 %	4.2 %	7.1 %
Winter	6.9%	3.6%	0.8 %	0.7 %	9.0 %	5.4 %	6.1 %	2.2 %	0.9 %	1.7 %

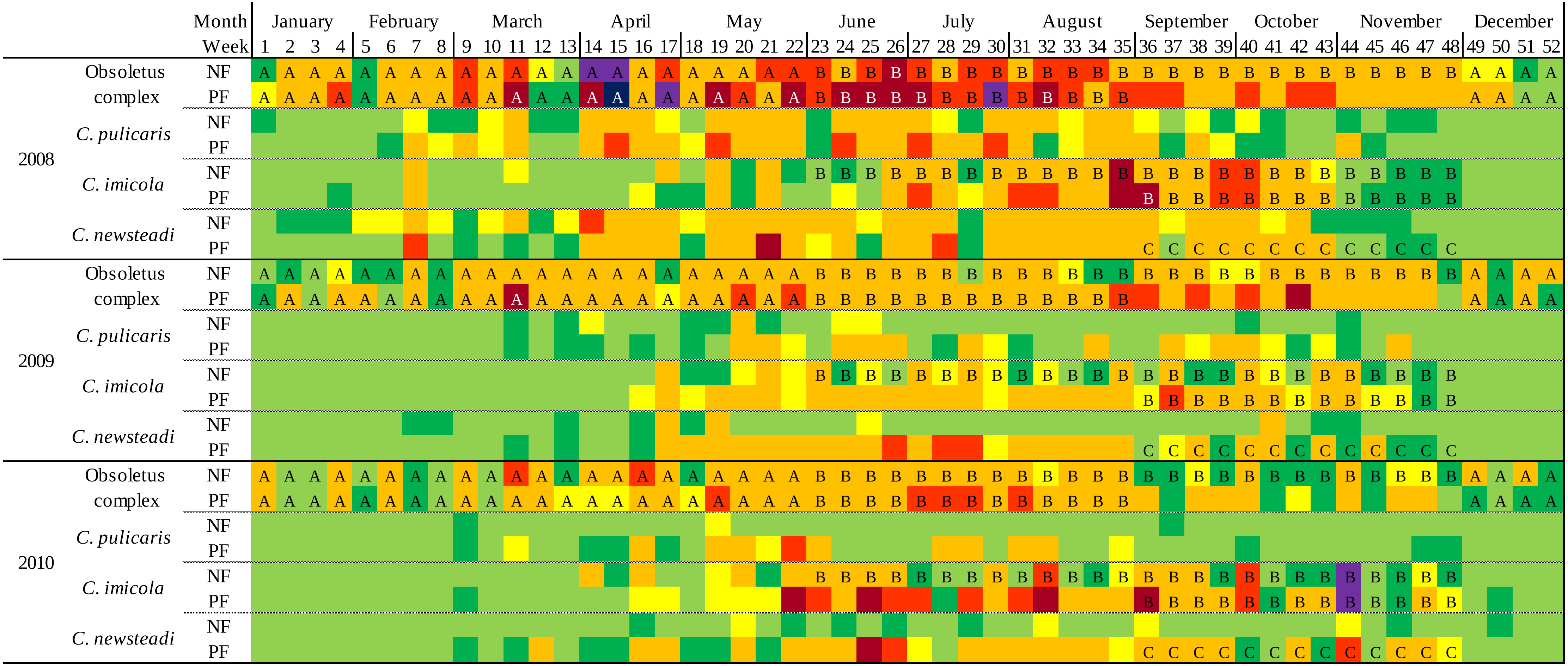


Figure 18. Mondrian matrix of the weekly maximum catches of nulliparous (NF) and parous females (PF) from 2008 to 2010 in mainland Spain (colour coded) for the species Obsoletus complex, *C. pulicaris*, *C. imicola* and *C. newsteadi*. Light green= 0 individuals, dark green= 1-4 individuals, yellow= 5-9, orange= 10-199, red= 200-499, dark red= 500-999, purple= 1000-2999, blue= 3000-5000. Letters indicate significant differences between species among seasons being (A): Significant differences respect the other species (2-talied K-S tests, *P*<0.05), (B): Significant differences respect to *C. newsteadi* and *C. pulicaris* (2-talied K-S tests, *P*<0.05), (C): Significant differences respect to *C. pulicaris* (2-talied K-S tests, *P*<0.05).

3.3.2. Seasonal pattern and abundance of *Culicoides* NF and PF from North to South axis in mainland Spain between 2008 and 2010.

The results of seasonal pattern of abundance of NF and PF from North to South axis in mainland Spain are exhibited in Figures 19 and 20 for each studied species.

Obsoletus complex: This species complex was found in all the provinces. The presence of Obsoletus complex species increased from south to north (with the exception of Toledo) with the *Culicoides* abundance being significantly higher in Northern provinces than the Southern ones (one-way K-W test, $P<0.05$) (Fig. 19). The province with the highest presence of Obsoletus complex was the Northern one, Navarra, where the species appeared during the whole year excepting February. The population abundance through the year in this province was significantly higher than in the other six provinces (2-tailed K-S tests, $P<0.05$), reaching the highest monthly maximum abundance of 668 PF collected per trap night in June 2009. Meanwhile, Ciudad Real was the province with the lowest number of individuals captured, with no more than 5 individuals recorded in June 2008.

The timing of the peaks of abundance varied between provinces. The peaks appeared earlier in Southern provinces, occurring in June in Cádiz; between June and July in Córdoba, Ciudad Real, Toledo and Guadalajara; and between July and September in Zaragoza and Navarra.

Southern provinces showed longer free-periods of this species. Guadalajara, Ciudad Real and Cádiz showed eleven months free of Obsoletus complex species followed by ten months in Córdoba, nine in Toledo, eight in Zaragoza and between one and six in Navarra. In fact, years 2009 and 2010 were almost free of this species during the whole year in Córdoba and Ciudad Real, and also Cádiz in 2010.

Culicoides pulicaris: This species was the one with the lowest abundance. As for the Obsoletus complex, the sites with the highest abundance of *C. pulicaris* were Navarra and Toledo. Navarra showed the highest monthly maximum abundance of 328 PF per trap night recorded in April 2009, whereas Toledo showed the longest seasonal adult period of *C. pulicaris* in 2008 with only 4 months free of that species. These two provinces showed significant higher PF abundance than the other five provinces (2-talied K-S tests, $P<0.05$), however, no clear differences in abundance were observed between Northern and Southern provinces (one-way K-W test, $P>0.05$).

Navarra, Zaragoza and Cádiz had an abnormally short duration of the *C. pulicaris* adult period. Navarra exhibited 11 months almost free of PF of this species during 2008; Zaragoza was almost free of *C. pulicaris* during the whole 2009 and 2010 and also free in Cádiz during 2010.

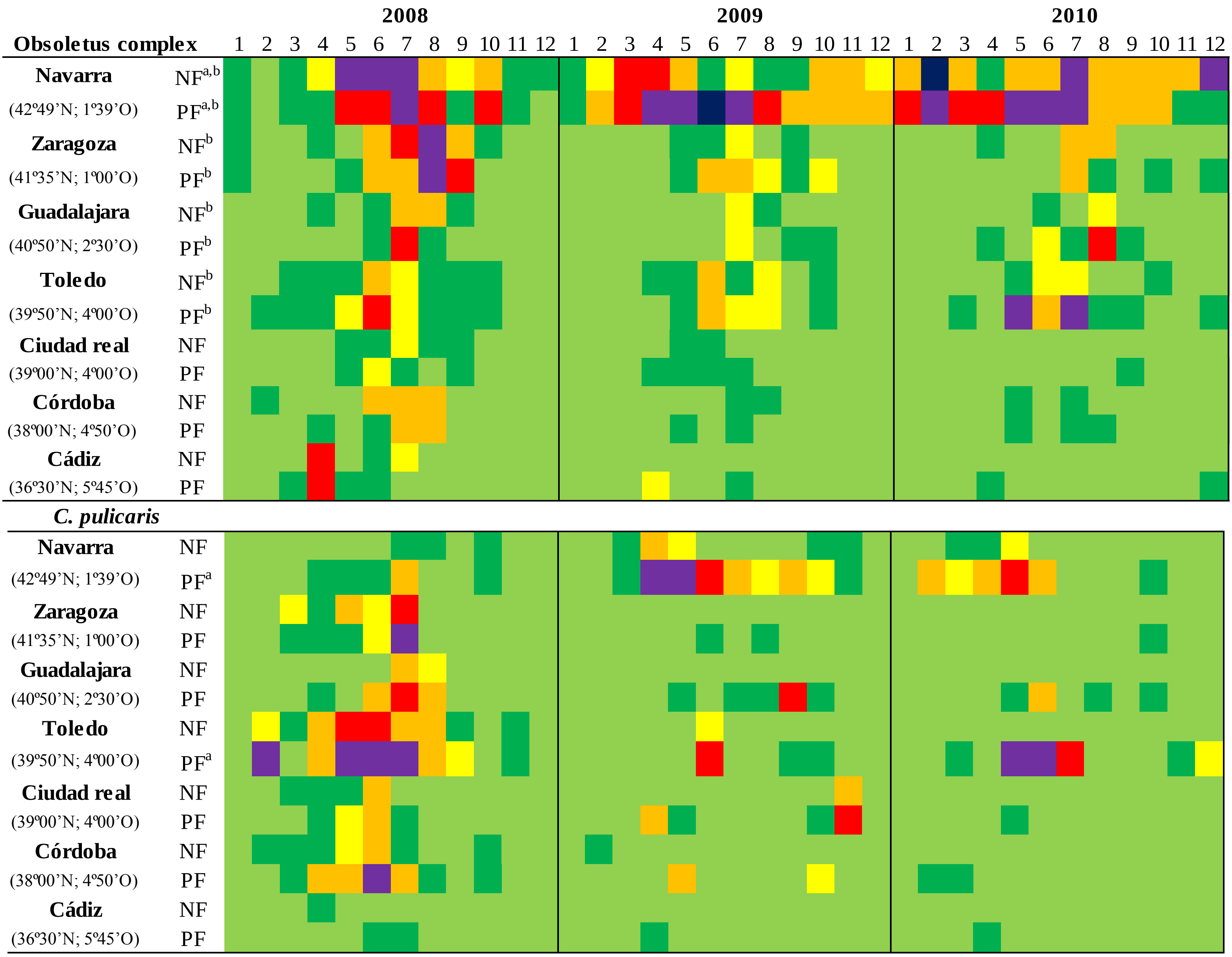


Figure 19. Mondrian matrix of the monthly maximum catches of nulliparous (NF) and parous females (PF) from 2008 to 2010 between the Northern province (Navarra) and the Southern one (Cádiz) (colour coded) for the species Obsoletus complex (above) and *C. pulicaris* (bellow). Green= 0 individuals, dark green= 1-4, yellow= 5-9, orange= 10-39, red= 40-99, purple= 100-499, blue= 500-1000. Letters indicate significant differences among provinces being (a): Significant differences respect to the other six provinces (2-tailed K-S tests, P<0.05), (b): Significant differences respect to the Southern provinces (2-talied K-S tests, P<0.05).

***Culicoides imicola*:** This species was absent in the Northern provinces of Navarra and Zaragoza and few specimens were captured in Guadalajara, in fact, no significant differences in abundance were found between these three Northern provinces (one-way K-W test, $P>0.05$, Fig. 20). Toledo was the most Northerly province with *C. imicola* captures and the one with the highest abundance; reaching a maximum monthly abundance of 718 PF on July 2010. In fact, Toledo exhibited significant differences in abundance with respect to the other six provinces (2-tailed K-S tests, $P<0.05$).

Although the variations in seasonality across the three years, *C. imicola* PF were usually absent from December to March in the Southern provinces of Ciudad Real, Córdoba and Cádiz. No significant differences in abundance were found between these three provinces (one-way K-W test, $P>0.05$), with the exception of Córdoba where PF abundance was significantly higher than Cádiz (2-tailed K-S tests, $P<0.05$). Cadiz province showed an abnormal results in 2010 where *C. imicola* adults only appeared in October.

Culicoides newsteadi: As for *C. imicola*, the highest population of this species was found in Toledo, reaching a maximum monthly abundance of 789 PF per trap night in May 2009 and with just one month (January) with completely absence of PF of this species. In addition, Toledo populations were significantly more abundant that in the other six provinces (2-tailed K-S tests, $P<0.05$, Fig. 20). Meanwhile, the remaining provinces (with the exception of Ciudad Real) recorded captures lower than 100 PF per trap and night during the whole year. Córdoba in 2009, Navarra in 2010 and also Cádiz in 2009 and 2010 showed the longest period of absence of *C. newsteadi* (11 months). However, no significant differences in abundance were found between Southern and Northern provinces (one-way K-W test, $P>0.05$).

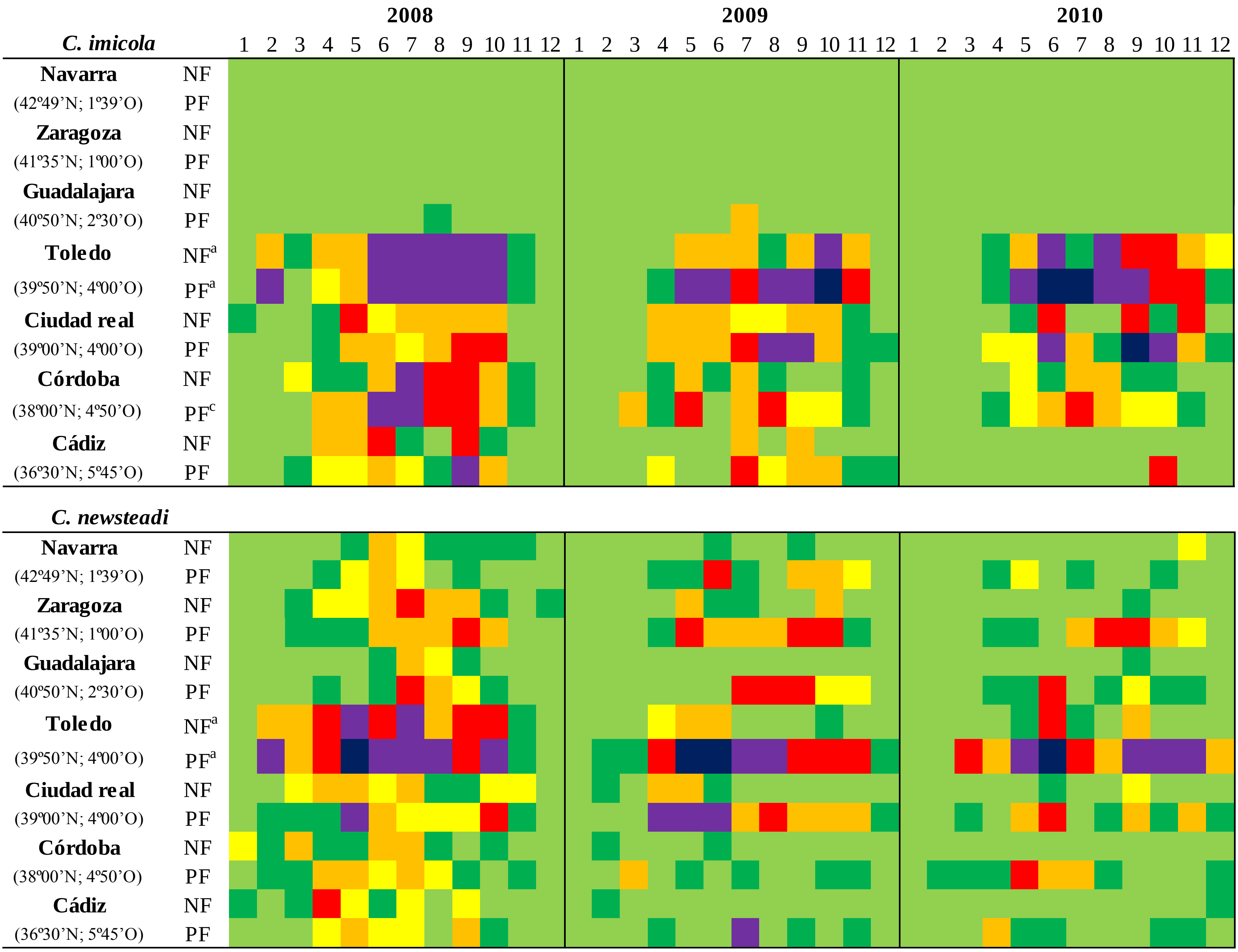


Figure 20. Mondrian matrix of the monthly maximum catches of nulliparous (NF) and parous females (PF) from 2008 to 2010 between the Northern province (Navarra) and the Southern one (Cádiz) (colour coded) for the species *C. imicola* (above) and *C. newsteadi* (bellow). Green= 0 individuals, dark green= 1-4, yellow= 5-9, orange= 10-39, red= 40-99, purple= 100-499, blue= 500-1000. Letters indicate significant differences among provinces being (a): Significant differences with respect to the other provinces (2-talied K-S tests, *P*<0.05), (c): Significant differences with respect to Cádiz province (2-talied K-S tests, *P*<0.05).

3.4. Discussion

3.4.1. General seasonal pattern and abundance of *Culicoides* NF and PF in Spain between 2008 and 2010.

Seasonality and abundance of adult female *Culicoides* are key parameters governing transmission of midge-borne viruses. A high ratio of adult vectors to hosts increases the basic reproduction ratio (R_0) (Diekmann and Heesterbeek 2000, Keeling and Grenfell 2000), so high adult vector population is a pre-requisite for autochthonous local transmission after a BTV introduction. Furthermore, the length of the SVFP over winter determines whether cycles of BTV transmission can be maintained between years since the maximum length of viraemia in the vertebrate host lasted only 9 weeks in adult cattle (OIE 2014).

This is the first analysis of the seasonal trend of PF at national level in Spain. For all the taxa studied, the season that contained the highest proportion of the annual count of PF was summer, which always encompassed over 50% of individuals. When the proportion of PF is higher than the NF, the risk of transmission of BTV increases. In the USA and South Africa, parity was used to predict disease transmission (Venter *et al*. 1997, Mayo *et al*. 2014), whereas in Canada the data of PF was used for understanding adult survival rates (Lysyk 2007). As mentioned previously, in Europe, only studies conducted in Czech Republic (Rádrová *et al*. 2015) and Italy (Foxi *et al*. 2016) included PF and non-parous annual proportions to understanding voltinism of vector species; so, there is a lack of information available about the relationship of NF/PF ratios with the seasonal occurrence of BTV outbreaks.

PF of the four species studied in the present work were detected during the three year surveillance. PF of Obsoletus complex were more abundant during summer, but were even present in winter. Though the seasonal pattern of this species may vary from year to year, the activity of PF of Obsoletus complex in Spain did not cease during winter and may affect the potential for BTV transmission during cold seasons. The last week of December was the only period considered to be a SVFP among the three years, not enough to break the cycle of transmission. This year-round activity has also been highlighted in Corsica where two species from the Obsoletus complex (*C. obsoletus* and *C. scoticus*) can be found all year (Venail *et al*. 2012). In fact, vectorial capacities of this species are a serious hazard since *C. obsoletus* followed by *C. scoticus* are the most abundant species from this group in Spain (Sarto I

Monteys and Saiz-Ardanaz 2003, Pagès and Sarto I Monteys 2005, Alarcón-Elbal *et al*. 2016, Kluiters *et al*. 2016).

Conversely, *C. pulicaris*, *C. imicola* and *C. newsteadi* were practically absent during winter, indicating that winter can be considered to be a free-period for these three species in Spain. The activity of *C. imicola* females, the main BTV vector in the Mediterranean Basin, during winter months has been previously cited in Portugal (Capela *et al*. 2003); however, that study did not include information regarding the age grade of captured females. The results obtained from this species coincided with those obtained in the Balearic Islands (Miranda *et al*. 2008), where, after analysing data from 2004 to 2007, the highest proportion of *C. imicola* PF was found also in summer (40 % of the total annual capture).

There were differences in the timing of the seasonal peak of abundance among the *Culicoides* species included in this work. Obsoletus complex, *C. pulicaris* and *C. newsteadi* had the main peak of PF located between spring and summer. Meanwhile, PF of *C. imicola* peaked in summer and autumn coinciding with the historical season of higher transmission of BTV in the Mediterranean Basin (reviewed in Jacquet *et al*. 2015). That differences coincided with previous works of the seasonal distribution of the total females (not age graded) in the Balearic Islands, mainland Spain, Portugal, Mediterranean coast of France, mainland Italy, Corsica, Sicily, Sardinia and UK (Ortega *et al*. 1997, 1998, 1999, Capela *et al*. 2003, Miranda *et al*. 2004, Purse *et al*. 2004b, Torina *et al*. 2004, Conte *et al*. 2007, Gerbier *et al*. 2008, Foxi and Delrio 2010, Sanders *et al*. 2011, Venail *et al*. 2012).

Culicoides pulicaris and *C. newsteadi* showed lower abundance compared to Obsoletus complex and *C. imicola*. However, these species are particularly interesting since BTV has been isolated/detected from field specimens (Goffredo *et al*. 2015, Foxi *et al*. 2016) and an increase of their abundance in the Mediterranean Basin may play an important role on BTV transmission (Torina *et al*. 2004, Foxi and Delrio 2010).

Species included in the Obsoletus complex were not identified and counted separately (Garros *et al*. 2014, Harrup *et al*. 2015); so, the seasonality of the different species and their particular level of implication with BTV transmission cannot be assessed here. Studies conducted in UK, Germany, France and Italy used molecular techniques or the morphology of male genitalias for separating the species *C. obsoletus* and *C. scoticus*: These studies have shown differences in the seasonal abundance of both species. In UK, *C. scoticus* season was eight weeks shorter than *C. obsoletus* (Searle *et al*. 2014). In Italy, *C. scoticus* was more abundant from late winter to early spring whereas *C. obsoletus* was more abundant in early summer; otherwise, in Germany, *C. scoticus* was significantly more abundant in summer and autumn than *C. obsoletus* (Balczun *et al*. 2009, Pili *et al*. 2010). In our case, the Obsoletus complex species (*C. obsoletus*/*C. scoticus*) were more abundant in spring in agreement to the aforementioned results recorded by that of Venail *et al*. (2012) in France.

3.4.2. Seasonal pattern and abundance of *Culicoides* NF and PF from North to South axis in mainland Spain. Years 2008-2010.

The results showed that the Northern provinces Navarra, Zaragoza and Guadalajara exhibited the longest SVFP with five months of absence of *C. imicola* and less than 5 PF of Obsoletus complex species, *C. pulicaris* and *C. newsteadi*.

The months of activity and abundance of PF increased from South to North in case of Obsoletus complex species and decreased in case of *C. imicola*. This distribution was similar to the number of total females obtained by Rawlings and Mellor (1994), Talavera *et al*. (2015) and Cuéllar *et al*. (2018b). According to this, the period of risk of transmission of BTV in Northern provinces of Spain such as Navarra, Zaragoza and Guadalajara would be related almost exclusively to Obsoletus complex. These results coincided with more than 2,000 outbreaks of BTV-1 in Northern provinces including Navarra in 2008 (MAPA 2019). So, animal movement restrictions and vaccination could be mainly focused in spring in Northern provinces since the first seasonal peak of Obsoletus complex is located between April and May.

The species *C. imicola* was absent in all the provinces above Toledo, except in case of Guadalajara where few PF were captured in summer. Otherwise, PF of this species were very abundant in the south of Spain; so, this species would be then main vector of BTV in Southern localities. The species Obsoletus complex, *C. pulicaris* and *C. newsteadi* were less abundant in the south but could work in combination with *C. imicola*. However, the peaks of Obsoletus complex appeared earlier in Southern provinces, extending the vector period in these localities. In fact, high population of these species may be related with several outbreaks of BTV-8 occurred between 2008 and 2010 in Southern provinces, as well as BTV-4 outbreaks in Cádiz (MAPA 2019).

The limited distribution of *C. imicola* was clearly correlated to its Afrotropical requirements, showing lower adaptation to cooler North Spain climatic conditions (Guichard *et al*. 2014). In fact, authors such as Conte *et al*. (2003) and Wittmann *et al.* (2001) have found the isothermal of 9.7-12.5 ºC as the limit of distribution of this species in Europe. In addition, the limited ability of *C. imicola* pupae to survive in waterlogged habitats was also mentioned as a possible limiting factor for its Northern distribution (Calistri *et al*. 2003, Conte *et al*. 2007, Foxi and Delrio 2010). Climate change or episodes of high temperature (Niedbalski and Fitzner 2018) could increase the vector competence and modify the parous rate by accelerating the development and the feeding activity of this insect in cooler zones, increasing the risk of BTV transmission in border areas (Wittmann and Baylis 2000).

Culicoides pulicaris and *C. newsteadi* were always present in all the provinces but with low abundance, especially in southern zones (Talavera *et al*. 2015, Cuéllar *et al* 2018b). With the exception of Toledo, winter months may be considered a free-period for these two species and the Southern province, Cádiz, was almost free of *C. pulicaris* within the three years. These two species exhibited a higher abundance in Toledo which is located in the middle of mainland Spain, overlapping with *C. imicola* and increasing the risk of BTV transmission. This fact could be related with inland preferences of these two species (De Liberato *et al*. 2003, Ducheyne *et al*. 2013), as well as low precipitation that characterize the province (AEMET 2018). However, there is still a lack of information about the environmental requirements of these two species. The high abundance of *C. imicola*, *C. pulicaris* and *C. newsteadi* suggests a stronger disease management effort in this overlap zone since these three species are potential vectors of BTV. Higher restrictions of animal movements and also an increase of vaccination campaigns may be considered.

The abnormal and unexpected cases of low population densities in 2008 recorded for *C. pulicaris* in Navarra and also the few presence of *C. imicola* in Cádiz on 2010 could be related to climate fluctuations among the years. In fact, mean temperatures in 2008 were extremely high in the North and year 2010 showed abnormal high precipitations in the south (AEMET 2019). These episodes of increase of temperature in the north could benefit the spread of cold-adapted *Culicoides*, whereas high precipitation in the south may affect negatively to *C. imicola*, due to their aforementioned preference for no-waterlogged areas. In addition, parameters such as temperature, land use and landscape connectivity determine the microhabitat availability, development, survival and feeding activity of the different *Culicoides* species, which may explain the differences between sites at same latitudes and between years (Searle *et al*. 2014). These data should be also considered as an additional criteria for the definition of the SVFP (EFSA 2017).

In order to improve the results of this study, further analysis including more provinces would be interesting to determine climatic parameters, topography, land use, host densities, habitat availability, etc. as factors in transmission risk assessment. Calvete *et al*. (2008) used climatic parameters to determine the special coincidence of Obsoletus complex and *C. imicola* in Spain; thus, future studies including the other species *C. pulicaris* and *C. newsteadi* and other Mediterranean countries in the analysis would be of interest by observing the interaction between the proximity from the coast and the *Culicoides* seasonality. All focused for a better understanding the role of the seasonal trend of PF in BTV occurrences.

3.5. Conclusions

The SVFP analysing the general seasonality of *Culicoides* in Spain was located in the last two weeks of December. Obsoletus complex were present and abundant within the whole year in Spain and especially in Northern provinces. *Culicoides pulicaris* and *C. newsteadi* were less abundant and have defined periods of absence lasting eleven months.

In agreement with Calvete *et al*. (2008) and posterior studies such as Ducheyne *et al*. (2013), this work has also demonstrated that Northern provinces, Navarra and Zaragoza were virtually free of BTV transmission risk due to *C. imicola*. Winter months (December-March) were considered SVFP in all the seven provinces included in the present work, with the exception of Toledo where *C. newsteadi* PF may be present in December and February.

Culicoides species present in Spain allow BTV transmission throughout all seasons since temperatures are high enough for gonotrophic cycles continue and completion of the EIP. In fact, there is a positive correlation between temperature and the case-reproduction ratio (R_t). The R_t fell below the threshold at temperatures below 21° in Southern provinces and 15ºC in Norther provinces (Boender *et al*. 2014, Guichard *et al*. 2014, Napp *et al*. 2016). These differences in temperature were related to cold adaptions of the Northern species (e.g. Obsoletus complex) in relation with other species such as *C. imicola*. Hence, the abundance of PF of Obsoletus complex and *C. imicola* along with *C. pulicaris* and *C. newsteadi* within the year is enough to allow transmission cycles to continue year-round.

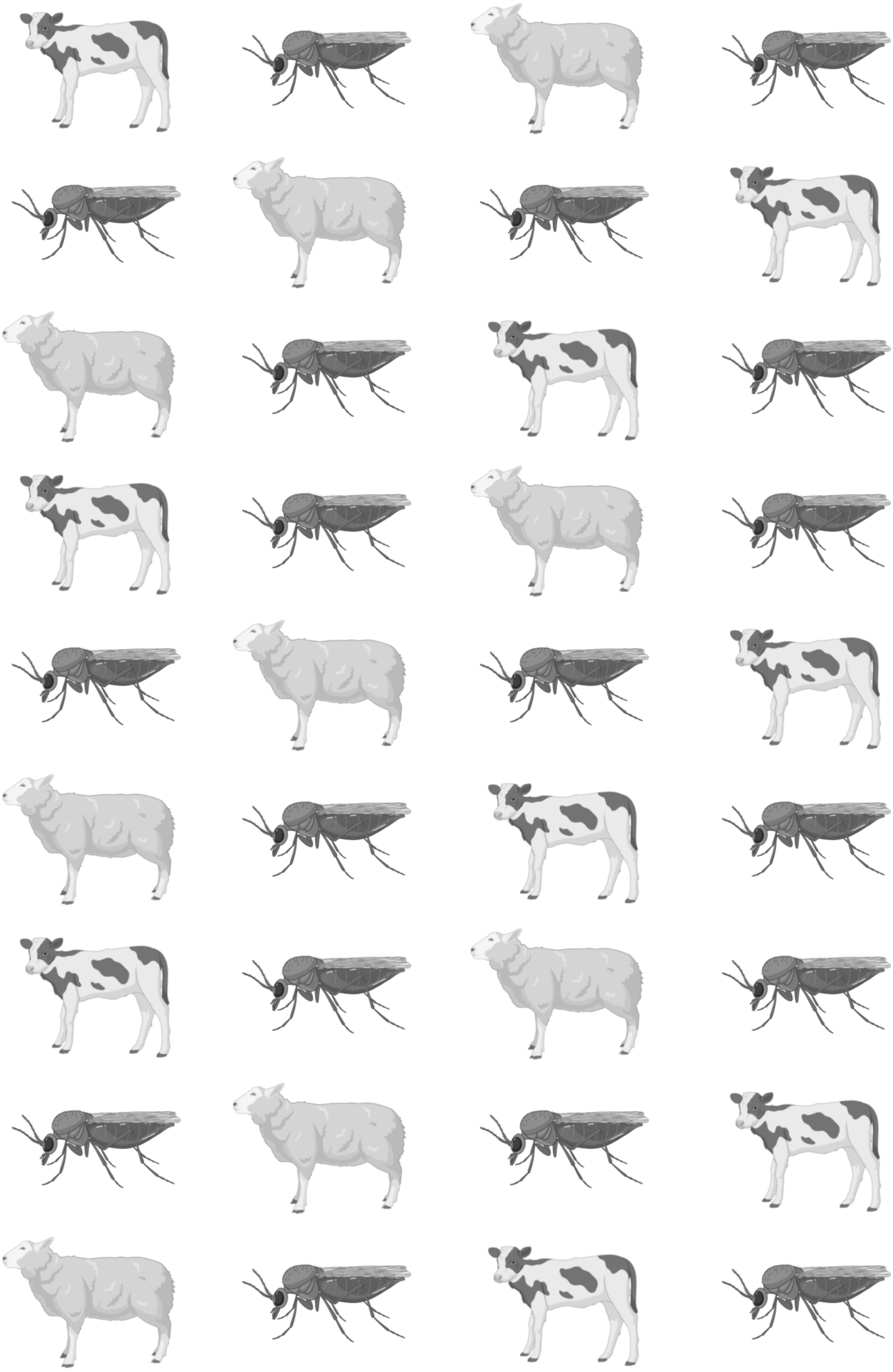
Determine the percentage of PF is a key factor to assess the risk of transmission of BTV. We have been able to obtain the data from the existing surveillance network, allowing us to make a first approximation to its relevance in the different provinces and seasons over the year. Our data have been used in other studies such as Calvete *et al*. (2006, 2009a, 2009b), Ducheyne *et al*. (2013) and Cuéllar *et al*. (2018a, 2018b); however, we have gone a step further by looking at the PF population of these four vector species. These data will be useful for a better approach in evaluations of BTV transmission risk assessments in Spain, a virus of veterinary and economic importance. Therefore, management in terms of when animal movements or vaccination campaigns could be planned.

The increase of PF mainly occurred in spring and summer for all the *Culicoides* species included in the present study, except in case of *C. imicola*. In fact, the highest abundance of PF was found between April and July for Obsoletus complex, *C. pulicaris* and *C. newsteadi* while was in November in case of *C. imicola*. This fact should be considered to incriminate the species responsible of the virus transmission during the BTV outbreaks since the species can be a bridge between seasons. For example, Obsoletus complex could start the transmission in spring followed by *C. imicola* in summer and autumn then overwintering in localities such as Toledo where can overlap its activity with *C. pulicaris* and *C. newsteadi*.

Climatic change that affects the Iberian Peninsula by increasing the minimum winter temperatures and the annual precipitation will influence the distribution of the species (e.g. the availability of moist breeding sites for these species), as well as duration of the SVFP. Some species such as *C. pulicaris* and *C. newsteadi* that are not currently active throughout the year may increase their adult activity period, leading to an increase in their corresponding vector capacity; however, the reduction of their breeding sites could limit their expansion to other locations and broad-scale analyses alone are insufficient to understand the potential impacts of changes in climate on arbovirus vector populations (Sanders *et al*. 2019).

The monitoring of these species will continue to be considered a fundamental task to deal with future BTV outbreaks in Spain. 31 permanent traps are currently working during the whole year in Spain considering four zones: the north (*C. obsoletus* main distribution, 13 sampling points), the south (*C. imicola* main distribution, 7 sampling points), the centre (overlapping area of *C. obsoletus* and *C. imicola*, 7 sampling points) and the Canary (2 sampling points) and Balearic islands (2 sampling points) archipelagos (MAPA 2019). The data from these samples are used for determining the SVFP. However, further research including *Culicoides* behaviour, vector control methods and transmission containment measures should be considered, especially where multiple potential vector species occur together.

4. Environmental drivers of seasonal activity and abundance of bluetongue vector species in Spain



4. Environmental drivers of seasonal activity and abundance of bluetongue vector species in Spain

4.1. Introduction

Bluetongue (BT) as well as Schmallenberg and African Horse Sickness are viral infectious diseases of ruminants (Taylor 1986, MacLachlan 1994, Gibbens 2012, Kauffold *et al*. 2014) transmitted by several species of biting midges *Culicoides* (Diptera; Ceratopogonidae) (Mellor *et al*. 2000, Purse *et al*. 2015).

BTV first appeared in Spain when serotype 10 arrived in 1956 (Sellers *et al*. 1978). Since then, circulation of 5 different serotypes (BTV-1, 2, 4, 8 and 10) has been detected during different periods. The major source of incursions of BTV strains is considered to be via infected *Culicoides* transported on the wind from North Africa (Zientara and Sánchez-Vizcaíno 2013, Pérez de Diego *et al*. 2014). Another potential introduction method of this virus is animal movements across Europe. Since 1956, more than 150,000 domestic animals were lost during BTV outbreaks in Spain, with devastating impact on the livelihoods of the farmers (Alba *et al*. 2004, Pérez de Diego *et al*. 2014). The last outbreak of BTV in Spain was in 2015 when BTV serotype 1 was detected in a goat farm located in the province of Cadiz (RASVE 2016). Currently, the outbreaks are restricted to serotype 4 in the centre of Iberian Peninsula and serotypes 1 and 4 in central and south areas (EU 2019).

Seasonality and abundance of adult female *Culicoides* are key parameters governing transmission of midge-borne viruses because a high ratio of adult vectors to hosts increases the basic reproduction ratio (R_0) (Diekmann and Heesterbeek 2000, Keeling and Grenfell 2000, Sanders *et al*. 2011). i.e. the probability that disease transmission will establish following introduction. Moreover, the length of the adult vector-free period over winter determines whether BTV will persist between years since the maximum length of viraemia in the vertebrate host is only nine weeks (in adult cattle) (OIE 2014). As a consequence, in temperate areas like Europe, disease management policy is governed by knowledge of the timing of this period (e.g. Seasonal Vector Free Period, or SVFP, defined in Annexe V of Commission Regulation (EC) No. 1266/2007 by the European Union council). Both vaccination campaigns and animal movements between affected regions can be conducted within the SVFP. This period is also used as a parameter in models aiming to understand the behaviour of virus outbreaks across geographical areas and, to assess effectiveness of control measures to control the virus in Spain (Gubbins *et al*. 2008, Hartemink *et al*. 2009, Brugger and Rubel 2013, Brugger *et al*. 2016, Napp *et al*. 2016).

Larval development and reproduction of adult vectors can occur year round in tropical areas and adults may be active during the whole year (Sellers 1980, Verhoef *et al*. 2014). However, in temperate and cool regions, *Culicoides* overwinter at larval stage and some adult species enter into diapause probably when the number of daylight hours or the temperature declines below a threshold level (Rawlings and Mellor 1994, Lysyk and Dank 2007), however, these links have not been explicitly quantified for any *Culicoides* species.

In Spain, the transmission of Bluetongue virus (BTV) is mainly attributed to the species *C. imicola* and the Obsoletus complex (Braverman *et al*. 1985, Mellor and Wittmann 2002, Purse *et al*. 2007, Wilson and Mellor 2009) (Table 2). The Obsoletus complex include *C. obsoletus*, *C. scoticus*, *C. chiopterus*, *C. montanus* and *C. dewulfi* (Boorman *et al*. 1995, Monaco *et al*. 2010, Garros *et al*. 2014), however, *C. obsoletus* and *C. scoticus* are probably one of the commonest group of *Culicoides* species across the whole of central and northern Europe (Rawlings and Mellor 1994, Hoffmann *et al*. 2008, Meiswinkel *et al*. 2008). In Spain, this taxa is found mainly in the North (Calvete *et al*. 2008, Lucientes *et al*. 2008, Calvete *et al*. 2009b, Talavera *et al*. 2015, Alarcón-Elbal *et al*. 2016) whereas the Afrotropical species *C. imicola* may have expanded its range northwards in recent decades due to the climate change (Purse *et al*. 2005, Guichard *et al*. 2014, Niedbalski and Fitzner 2018), reaching Switzerland as the northernmost direction (Cagienard *et al*. 2006). In Spain, this species is still restricted in south-western parts including the Mediterranean Basin (Ortega *et al*. 1998, Tatem *et al*. 2003, Calvete *et al*. 2008, 2009a, Acevedo *et al*. 2010, Talavera *et al*. 2015, Cuéllar *et al*. 2018b).

Recently, other species, namely *C. pulicaris* and *C. newsteadi*, have also been incriminated as a vector following the isolation of BTV from field specimens (Table 3). *Culicoides pulicaris* species is less prevalent than the others but shares a similar overall distribution to the Obsoletus complex in Spain (Lucientes *et al*. 2008, Ducheyne *et al*. 2013, Talavera *et al*. 2015), whereas *C. newsteadi* has a high population density in Spain and is distributed in the south (Ortega *et al*. 1999, Del Río *et al*. 2013, Ducheyne *et al*. 2013).

Table 3. Summary of vector species incriminated as vectors in Spain and their distribution. *Culicoides* populations and virus strains from other continents are not included.

Species	References (from Europe)	Incriminated as vector	Distribution
Obsoletus complex	(Mellor and Pitzolis 1979, Jennings and Mellor 1988, Savini *et al*. 2005, De Liberato *et al*. 2005, Carpenter *et al*. 2006, Meiswinkel *et al*. 2007, Carpenter *et al*. 2008a, Dijkstra *et al*. 2008, Vanbinst *et al*. 2009)	• BTV isolated from parous females pools • Laboratory infection and evidence of replication • Significant abundance in proximity to susceptible hosts	Palaearctic region
C. imicola	(Mellor and Pitzolis 1979, Ferrari *et al*. 2005, Del Río *et al*. 2012, Goffredo *et al*. 2015, Foxi *et al*. 2016).		Palaearctic (restricted in Mediterranean basin) and Afrotropical regions
C. pulicaris	(Jennings and Mellor 1988, Caracappa *et al*. 2003, Torina *et al*. 2004, Ferrari *et al*. 2005, Carpenter *et al*. 2006, Vanbinst *et al*. 2009, Goffredo *et al*. 2015, Foxi *et al*. 2016)		Palearctic and Afrotropical regions
C. newsteadi	(Goffredo *et al*. 2015, Foxi *et al*. 2016)	• BTV isolated from parous females pools	Palaearctic region

The objective of this study is to relate variability in the adult seasonality of the most important vector species of *Culicoides* present in Spain in different sites and years to concurrent host, landscape and climate conditions with the aim of predicting variation in the SVFP in different environmental contexts. We look at *Culicoides* adult females because only females take blood meals and lay eggs, making them the vector and dispersal stage of these taxa. The use of Bayesian Generalised Linear Mixed Model (GLMM) frameworks enables us to account for spatial correlation between sites and temporal correlation between years. A Wrapped Normal distribution (WN) was used to model the timing of appearance and disappearance, to account for the circular nature of such phenological event data.

The following specific associations are tested:

We assume that major environmental factors affecting development, activity and distribution of *Culicoides* populations in Europe are temperature, humidity and rainfall due to the use of moist/soil breeding sites (Harrup *et al*. 2013, Zimmer *et al*. 2013a, 2014b, Purse *et al*. 2015). In addition, prior studies established a direct relationship between *Culicoides* abundance measured in traps and the probability of BTV occurrences (Calvete *et al*. 2008, Guis *et al*. 2012). In agreement to the literature summarized in the Annex 1 and the previous chapter of the current thesis, *C. imicola* is highly abundant in South-western sites of Spain during summer; has longer seasons and prefer high temperatures, probably related with its Afrotropical preferences (Ortega *et al*. 1999, Miranda *et al*. 2004, Purse *et al*. 2004a, 2007, Talavera *et al*. 2015, Ramilo *et al*. 2017). Its peaks of adult activity are situated between the 16ºC and 18ºC and the species is absent in sites with cold winters (Rawlings and Mellor 1994, Venter *et al*. 2019). According to its helophilic behavior, this species breed in grassland areas with high vegetation temperature including wet but no-waterlogged coastal sites (Braverman *et al*. 1974, Mellor and Pitzolis 1979, Conte *et al*. 2007, Peters *et al* 2014a, 2014b) and lower altitudes (Conte *et al*. 2003, Torina *et al*. 2004, Conte et el. 2007). Otherwise, Obsoletus complex are favoured by lower average temperatures (Miranda *et al*. 2004, Conte *et al*. 2007, Talavera *et al*. 2015, Cuéllar *et al*. 2018a) and arid sites with high elevation and dense vegetation including forests since some members breed in lead-litter (Zimmer *et al*. 2013a, Torina *et al*. 2004, Conte *et al*. 2007, Carpenter *et al*. 2008b). Both species breed in habitats enriched with organic matter near livestock animals (Capela *et al*. 1993, Conte *et al*. 2007, Takken *et al*. 2008, Foxi and Delrio 2010, González *et al*. 2013b, Zimmer *et al*. 2014a, 2014b, Talavera *et al*. 2015).

As well as *C. imicola*, the species *C. newsteadi* species has also been found to prefer lower altitudes (Torina *et al*. 2004) and sites with lower average temperatures (Ortega *et al*. 1999, Purse *et al*. 2004a). *Culicoides pulicaris* has shown preference for the soil of open pastures (Harrup *et al*. 2013, Zimmer *et al*. 2014b) and appears in the same sites as Obsoletus complex (Ortega *et al*. 1998, Ducheyne *et al*. 2013) and also in coastal zones (Ramilo *et al*. 2017). Obsoletus complex and *C. pulicaris* could overwinter in stables during winter (Kameke *et al*. 2017), therefore, *C. pulicaris* is expected to be related to the same variables as Obsoletus complex such as lower temperatures and livestock (Purse *et al*. 2004a, Sanders *et al*. 2011, Searle *et al*. 2012).

In this study, we analyze the variation in phenology of adult females of populations of the following species: Obsoletus complex, *C. imicola*, *C. newsteadi* and *C. pulicaris* according to different environmental variables. The objective is to facilitate understanding of how the environment could affect these species and explore the contrast between the northern species Obsoletus complex/ C. pulicaris and the south-eastern species *C. imicola*/ *C. newsteadi*.

4.2. Material and methods

4.2.1. Entomological data and sampling procedures

Data used for the study were samples from 329 trap sites distributed in the 50 provinces of Spain (Fig. 21) during the intensive National Entomosurveillance Program for BTV sponsored by the Spanish Ministry of Rural and Marine Environment from year 2005 to 2010.

Figure 21. Map with the location of sampling points during the National surveillance program from 2005 to 2010.

Culicoides were collected by CDC-Miniature traps (John W. Hock®) with UV light and photoelectric cell. The traps were set one night per week on farms at 1.7-2.0 metres from the floor and placed between 1 and 30 metres from the livestock of domestic animals from dusk till dawn (at least 45 weeks per year). The collectors of the trap were bottles provided with antifreeze and alcohol to prevent the samples from decaying. The bottles were collected and transported to laboratory where they have classified by species and gonotrophic status according to Mathieu *et al*. (2012) taxonomic key and Dyce (1969). The farms were located at altitudes from 2 to 1456 meters above sea level and housed between 16 and 3000 large livestock animals including intensive farm settings (50% of all sites), 27% extensive and 23% semi-extensive farms. Amongst the farms, 60% were bovine farms, 26 % ovine farms, 6% equine farms, 5% caprine farms and 3% porcine farms.

All the data obtained from these sampling points were sent together to the reference laboratory located in the faculty of veterinary of Zaragoza province and data was sent to the Laboratory of Zoology (University of the Balearic Islands) for the analysis.

4.2.2. Models

The *Culicoides* taxa considered in this analysis were Obsoletus complex species, *C. imicola*, *C. pulicaris* and *C. newsteadi*. The species from Obsoletus complex are usually identified through molecular assays (Garros *et al*. 2014, Harrup *et al*. 2015), however, molecular methods were not used routinely during the surveillance program so the Obsoletus complex species were analysed together. Phenology metrics were only calculated for a particular site-year combination when sufficient trapping effort had been employed year round. This was defined as site-years with, at least, 45 trapping weeks per year and no more than 3 consecutive weeks with no-trapping. Since sampled population dynamics are inherently biased in small populations, for each species, we further excluded site-years in which a species occurred in very low average abundance for one or more two month periods of the year.

Six periods were defined as February and March (Period 1), April and May (Period 2), June and July (Period 3), August and September (Period 4), October and November (Period 5) and December and January (Period 6). The abundance thresholds that constituted low abundance were taxa-specific as follows: In case of Start and End of season models for Obsoletus complex- mean of 1 or less individuals per a trap catch; *C. pulicaris* and *C. newsteadi* - mean of 5 or less individuals per trap catch; *C. imicola* - mean of 20 individuals or less per trap catch. Regarding the length of overwinter models for Obsoletus complex, *C. pulicaris* and *C. newsteadi* - mean of 1 or less individuals per a trap catch; *C. imicola* - mean of 20 individuals or less per trap catch. The numbers of site-year combinations available in total and the numbers which met the above criteria for each taxa are shown in Table 4.

Table 4. Number of total data points and data points after species abundance thresholds had been applied. For start and end of season, thresholds were as follows: Obsoletus complex: >1 females / trap catch, for *C. newsteadi* and *C. pulicaris*: >5 females / trap catch and for *C. imicola* >20 females / trap catch. For length of overwinter period, thresholds were: Obsoletus complex, *C. newsteadi* and *C. pulicaris*: >1 females / trap catch and for *C. imicola* >20 females / trap catch. N: number of samples.

	Total datapoints			**Datapoints with threshold**		
Species	**N**	**Sites**	**Years**	**N**	**Sites**	**Years**
Start and End of the Season						
Obsoletus complex	106	61	6	84	54	6
C. imicola	92	48	6	57	37	6
C. newsteadi	93	50	6	58	38	5
C. pulicaris	81	44	6	40	25	5
Length of overwinter						
Obsoletus complex	47	31	5	38	29	5
C. imicola	46	26	5	29	18	5
C. newsteadi	44	26	5	35	23	4
C. pulicaris	42	27	4	35	25	4

To test links between adult seasonality and environmental factors, we calculated the following metrics of annual abundance and seasonality for each year in each site:

- Start of the season (Julian days): Defined as the first day of the year in which more than one (in case of Obsoletus complex), five (in case of *C. newsteadi* and *C. pulicaris*) or 20 (in case of *C. imicola*) females were caught.
- End of the season (Julian days): Defined as the last day of the year in which more than one (in case of Obsoletus complex), five (in case of *C. newsteadi* and *C. pulicaris*) or 20 (in case of *C. imicola*) females were caught.
- Length of the overwinter period (in days): Defined as the difference in days between the end of the season in one year and the start of the season of the following year.

4.2.2. Environmental parameters

Environmental data of each site were obtained from different sources such as CORINE, SRTM, ENSEMBLES and Robinson *et al*. (2012) and used to perform the models in order to see the negative or positive environmental effects on *Culicoides* populations. The environmental data used were: Topography (elevation and slope); the most predominant classes of CORINE land cover in Spain considered relevant to *Culicoides* occurrence included pastures, natural grassland zones, agro-forestry areas, schlerophyllous vegetation (type of vegetation adapted to cope with hot and dry summers), broad-leaved forest and mixed forest; climate factors (accumulate degree days and rainfall for each season); photoperiod (in March, April, September and November); host density (cattle and sheep in a 2-km grid square) and an estimate of the total *Culicoides* female abundance of each species at each site in each year (Table 5). This last parameter was used because the timing of emergence and disappearance will be sampled more accurately in sites with higher abundances of *Culicoides* (Searle *et al*. 2012, 2014, Daoud 2017).

Table 5. Environmental variables included as potential predictors of patterns in *Culicoides* phenology in Spain (timing of start and end of season, and length of overwinter (OW)) extracted for each of the trapping site by year combinations.

Environmental data	Notation	Definition	Start	End	OW
Topography (source: SRTM 90m Digital Elevation Database v4.1, CGIAR-CSI. URL: http://www.cgiar-csi.org/data/srtm-90m-digital-elevation-database-v4-1)	Elev	Elevation in meters above the sea level at 90-m grid square resolution	X	X	X
	Slope	Inclination of the land in degrees at 90-m grid square resolution	X	X	X
Host density (source: Robinson *et al*. 2012)	Cattle	Number of cattle in a 2-km grid square for each trapping site.	X	X	X
	Sheep	Number of sheep 2-km grid square for each sampling point.	X	X	X
Land cover (source: Corine Land Cover 2006 vector; min.: 25 ha/polygon. URL: https://www.eea.europa.eu/data-and-maps/data/clc-2006-vector-4).	NatGras	Percentage of Natural grassland zones in the surrounding 1 km around each trapping site.	X	X	X
	PastGras	Percentage of Pasture grassland zones in the surrounding 1 km around each trapping site.	X	X	X
	AgFor	Percentage of Agro-forestry areas in the surrounding 1 km around each trapping site.	X	X	X
	SchVeg	Percentage of Schlerophyllous vegetation in the surrounding 1km around each trapping site.	X	X	X
	BrdMix	Broad leaved forest and mixed forest in the surrounding 1km around each trapping site.	X	X	X

Table 5. (Continued)

Environmental data	Notation	Definition	Start	End	OW
Temperature (source: ENSEMBLES.URL: http://ensembles-eu.metoffice.com/**)**	DDwin	Accumulated degree days greater than 10°C between December and March.	X		X
	DDaut	Accumulated degree days greater than 10°C between September and December.		X	X
	DDspr	Accumulated degree days greater than 10°C between March and June.	X		
	DDsum	Accumulated degree days greater than 10°C between June and September.		X	
Precipitation (source: EU-FP6 project ENSEMBLES. URL: http://ensembles-eu.metoffice.com/**)**	Pwin	Summed daily precipitation (mm) over 1st December to February 31st	X		X
	Paut	Summed daily precipitation (mm) over September 1st to November 31st		X	X
	Pspr	Summed daily precipitation (mm) over March 1st to May 31st	X		
	Psum	Summed daily precipitation (mm) over June 1st to August 31st		X	
Photoperiod (source CEH models)	Phmarch	Days in March with more than 9 hours of daylight.	X		
	Phapr	Days in April with more than 9 hours of daylight.	X		
	Phsep	Days in September with more than 9 hours of daylight.		X	X
	Phnov	Days in November with more than 9 hours of daylight.		X	X
Females abundance	Sum females	Mean annual average abundance of *Culicoides* females for a site.	X	X	X

4.2.3. Statistical methods

The relationship between the "start" and the "end" timings and the environmental drivers were quantified using Generalised Linear Mixed Model (GLMM) using circular regression obtained by wrapping a normal distribution on the real line around a circle (Modlin *et al.* 2012). It is a "generalised" model because it uses some statistical distributions that are not normally distributed; "linear" because it uses a linear combination of explanatory variables and structural components and a "mixed model" because it contains both fixed (the regression parameters like environmental variables) and random effects (the year and site parameters). Also, we have used a circular regression because the weeks for the "start" and "end" of the season are circular variables because they range from day 1 to 365 and days at the beginning and end of the year are close in time, so, we cannot assume that they have a standard statistical distribution. In case of the length of overwinter period, however, we used a normal distribution.

Bayesian models were performed using WinBUGS platform (Lunn *et al*. 2000, 2009) on the R-Development Core Team 2009 software and fitted following the approach of Modlin *et al.* (2012).

The "start", "end" and "length of overwinter" were the dependent variables, the environmental parameters were considered to be fixed effects and random effects for site and year were also added. A preliminary saturated model (with all environmental parameters) was first run in order to check that environmental models do better than a model without environmental variables. Then, all environmental parameters (fixed effects) were selected in subsets of broad categories and combined, given that they resulted in an improvement in model fit. The order of the broad categories added to the models was: female abundance, temperature, topography, photoperiod, precipitation, interactions between temperature and precipitation, hosts and land cover. A generalisation of the Akaike Information Criterion (AIC) called Deviance Information Criterion (DIC) (Spiegelhalter *et al*. 2002) was used as a criterion to find the most suitable model for each of timing metrics, by successively adding and removing environmental covariates from the board categories to find the model best supported by the data. Smaller values of DIC are indicative of better-fitting model and are derived as the mean deviance adjusted for the estimated number of parameters in the model supplying a relative measure of out-of-sample predictive performance (Gelman and Hill 2007). Before model fitting, we first checked the collinearity between the variables in each model; Variance Inflation Factors (VIF) was also calculated at the end of the variable selection where values below 5 were considered as negligible collinearity (Daoud 2017).

4.3. Results

Obsoletus complex were the most prevalent species across the whole sites in Spain (N= 106, 61 sites across 6 years) (Table 4) followed by *C. newsteadi* (N=93, 50 sites across 5 years), *C. imicola* (N= 92, 48 sites across 6 years) and *C. pulicaris* (N= 81, 44 sites across 5 years). The total number of *Culicoides* females captured during the National Surveillance in sites with, at least, 45 trapping weeks per year and no more than 3 consecutive weeks with no-trapping, was 1,317,103 individuals. Of these individuals, *C. imicola* represented the 50.6% of the total captures (666,563 individuals) followed by Obsoletus complex species: 478,819 (36.3%), *C. newsteadi*: 115,783 (8.8%) and *C. pulicaris* 55,938 (4.3%). However, these values changed when the species thresholds were applied for the models and the total midges collected were 18,377 (mean 322.4 ± 602.9) for *C. imicola*; 7476 (mean 89.0 ± 171.2) for Obsoletus complex; 3477 (mean 59.9 ± 84.3) for *C. newsteadi* and 1853 (mean 46.3 ± 56.4) for *C. pulicaris* (Table 6).

Table 6. Total and mean number of *Culicoides* caught by site and year used in analysis with thresholds applied for Obsoletus complex: >1 females / trap catch, for *C. newsteadi* and *C. pulicaris*: >5 females / trap catch and for *C. imicola* >20 females / trap catch. N: number of samples. (*): No catches in 2005.

					Mean by year					
Species	**N**	**Sites**	**Years**	**Total (Mean ± S.D.)**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Obsoletus complex	84	54	6	7476 (89.9 ± 171.2)	76.1	121.8	104.5	73.8	74.2	69.4
C. imicola	57	37	6	18,377 (322.4 ± 602.9)	1332.7	223.1	309.3	245.1	63.2	126.4
C. newsteadi	58	38	5*	3477 (59.9 ± 84.3)	-	19.9	96.1	53.9	33.0	29.2
C. pulicaris	40	25	5*	1853 (46.3 ± 56.4)	-	26.8	86.4	31.6	8.1	19.6

The maximum nightly catch was 39,000 individuals for *C. imicola* in Cáceres in September 2005; 14,571 individuals of Obsoletus complex in Girona (41º 59’ 00’’ N; 2º 49’ 00’’ E) in July 2008; 6400 individuals of *C. newsteadi* Badajoz (38º 53’ 00’’ N; 6º 58’ 00’’ W) in March 2006 and 1174 individuals of *C. pulicaris* in Toledo (39º 52’ 00’’ N; 4º 02’ 00’’ W) in June 2007.

4.3.1. Comparing phenology between *Culicoides* taxa

The median length of the adult season was quite similar between the *Culicoides* taxa studied, with adults being absent overwinter for around 18-30 days in *C. imicola* (M_e= 22 days), between 16-30 days (M_e= 23) in case of *C. newsteadi* and longer in Obsoletus complex (5-32 days, M_e= 24.5) and *C. pulicaris* (21-35 days, M_e= 29) (Fig. 22, C). Despite this, there were substantial differences in the timing of the adult season between taxa. Obsoletus complex and *C. newsteadi* appeared earlier, followed by *C. pulicaris*, then *C. imicola* (Fig. 22, A). The timing of the end of the season was more variable across the samples sites and years than was the start of the season. In autumn, *C. pulicaris* disappeared first, followed closely by *C. newsteadi*. Obsoletus complex and *C. imicola* ended 4 weeks later in October (Fig. 22, B).

Across all sites the species appeared on average between May and July. Obsoletus complex showed the widest variability in start of the season within all the sites where records varied between the week 7 and 22 (M_e = 16) (Fig. 22, A). This species were also the ones which appear earlier followed by *C. newsteadi* (weeks 10-21, M_e = 15), *C. pulicaris* (weeks 13-21, M_e = 17) and *C. imicola* (weeks 15-26, M_e = 18). In fact, first appearance of Obsoletus complex species occurred on average in early April with the first records in early January within several years in Balearic Islands (39º 30’ 00’’ N; 3º 00’ 00’’ E), Girona, Navarra (42º 49’ 00’’ N; 1º 39’ 00’’ W) and Pontevedra (42º 26’ 01’’ N; 8º 38’ 51” W). Otherwise, the site with the latest start of Obsoletus complex female’s season was recorded in Arcos de la Frontera (Cádiz, 36º 44’ 54’’ N; 5º 48’ 23’’ W) on late September of 2005. *C. imicola* species was the latest ones to appear with an average of appearance in early July but first records also in early January (M_e = 18) in Balearic Islands (2008), Badajoz (2007) and Málaga (36º 43’ 00’’ N; 4º 25’ 00’’ W) in 2005 and 2006. Meanwhile, the latest first records for this species were in late November in Málaga on 2008.

Regarding the end of season, *C. pulicaris* showed the widest variability in end of season adult activity with the season ending between the week 30 and 44 (M_e = 39) in the majority of sites across Spain. Meanwhile, *C. imicola* was the species which ended its activity later, with the timing of the end of the season occurring variously between the week 41.5 and 47 (M_e = 45) (Fig. 22, B). Obsoletus complex species and *C. newsteadi* had similar end of season periods ranging between week 35-46 (M_e= 42) and 37-46 (M_e= 45) respectively. The last *Culicoides* captures were recorded between September and October across the whole sites and years. The earliest last captures were individuals of *C. pulicaris* recorded in Medina-Sidonia (Cádiz, 36º 27’ 28’’ N; 5º 55’ 38’’ W) on early April 2008 whereas *C. imicola* species were the ones who were active until December in Málaga, Cádiz, Badajoz, Mallorca (39º 37’ 00’’ N; 2º 59’ 00’’ E) and Menorca (39º 58’ 00’’ N; 4º 05’ 00’’ E).

In regard to the overwinter period, *C. newsteadi* followed by *C. pulicaris* were the ones with highest number of days overwintering reaching values of 72 and 60 days respectively in Lugo (43º 00’ 42’’ N; 7º 33’ 26’’ W) on 2007 for *C. newsteadi* and Guadalajara (40º 38’ 00’’ N; 3º 10’ 00’’ W) on 2008 for *C. pulicaris*.

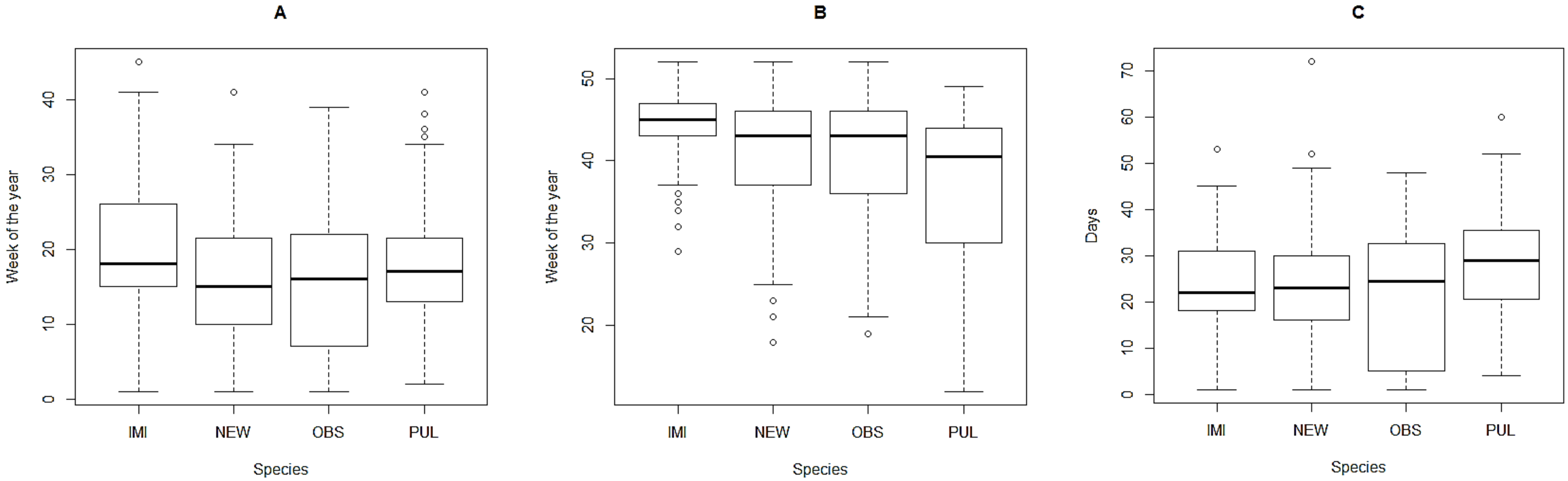


Figure 22. Differences between species in the timing of the start (A) and end (B) of seasonal activity (weeks of the year), and length of overwinter period (C; days) derived from Spain National Surveillance Program data during 2005 to 2010. Box plots show the median (central line), box denotes 25th and 75th percentiles, error bars represent 10th and 90th percentiles, and dots are points outside the 10th and 90th percentiles. Data are shown for *C. imicola* (IMI, N= 57), *C. newsteadi* (NEW, N=58), Obsoletus complex species (OBS, N= 84), and *C. pulicaris* (PUL, N= 40).

4.3.2. Factors affecting the start of season

The best-fitting model for the start of the season for Obsoletus complex (DIC= 180.93, annex 2) showed that the season started significantly earlier in sites with higher number of accumulated degree days over 10ºC in spring and when more days in April exceeded 9 hours of daylight. In addition, the season started significantly later when the elevation was higher (Table 7). The following seven top models with different environmental variables had the same ability to predict the data being within 2 DIC units from the best one (Annex 2).

No variables were included in *C. imicola* the best start of season model (DIC= 128.62), however, two models were within 2 DIC units from the model with no covariates (Annex 3).

The best model for the start of the season of *C. newsteadi* (DIC= 120.34, annex 4) indicated a significant negative effect of accumulated degree days greater than 10°C during winter, the number of sheep and the abundance of *Culicoides* females (Table 9). Therefore, the season of this species started earlier in places with high temperatures during winter, high number of sheep and high average annual abundance of the *Culicoides* population in the farm. The second best model with different environmental variables had the same ability to predict the data (Δ DIC < 2, annex 3).

In regard to *C. pulicaris* species, the best model (DIC= 88.77, annex 5) also showed a significant influence of the accumulated degree days greater than 10°C during winter (Table 10) which means that the season of this species started earlier in in places with high accumulated temperatures during winter. This variable was present and significant in the other top models that had Δ DIC < 2 (Annex 5) indicating an important impact on the start of the season.

4.3.3. Factors affecting the end of season

The best-fitting model for the end of the season of Obsoletus complex (DIC=180.15, annex 6) included a significant positive effect of cattle density (Table 7) which means that the season for that species ended later in sites with high abundance of cattle. Three other models showed similar support and the same ability to predict the data, being within 2 DIC units of the top model (Annex 6).

Culicoides imicola best model (DIC= 45.68, annex 7) included a significant negative effect of the elevation on the end of the season for that species (Table 8). Therefore, the season for that species ended earlier in sites with high elevation from the sea level. The eight other top models that had similar support in the data (Δ DIC < 2, annex 7) included elevation.

Regarding *C. newsteadi*, the predictors included in the best end of the season (DIC= 126.81, annex 8) were elevation, cattle livestock, photoperiod in September and abundance of *Culicoides* females, but none of these effects were significant (Table 9). Four top models showed similar support and same ability to predict the data, being within 2 DIC units of the top model (Annex 8).

Similarly, the best end of the season model for *C. pulicaris* (DIC= 68.33, annex 9) included accumulated degree days over 10ºC in summer, precipitation in summer, the relation between the accumulated degree days and the precipitation during summer, the abundance of sheep livestock, photoperiod in September and *Culicoides* females abundance (Table 10) but none of these effects were significant. The best model had the same ability to predict the data than the second best model due to its Δ DIC < 2 (Annex 9).

4.3.4. Factors affecting the length of overwinter

Obsoletus complex best overwinter model (DIC= 344.18, annex 10) showed that elevation had a significant positive effect on the length of overwinter while accumulated degree days over 10ºC and precipitation in autumn, the photoperiod in September and the *Culicoides* females population all had significant negative effects on that length of overwinter (Table 7). Therefore, sites at high elevation, those with low temperature and precipitation in autumn and less daylight hours in September or low abundances of *Culicoides* females had longer overwintering periods. The following two best models had similar support in the data, being within 2 DIC units of the best model (Annex 10).

The best overwinter model for *C. imicola* (DIC= 171.80, annex 11) showed that sites with more accumulated degree days over 10ºC in winter combined with high precipitation during that season had longer overwintering periods (Table 8).

The best-fitting overwinter model for *C. newsteadi* (DIC= 335.65, annex 12) indicated a significant effect of six environmental variables. The slope of the land and higher densities of cattle livestock had a positive significant effect on the length of overwinter whereas the accumulated degree days greater than 10ºC in autumn, the precipitation during winter, the percentage of agro-forestry areas and the schlerophyllous vegetation had a significant negative effect on this seasonal metric (Table 9). Hence, sites with longer periods of days over 10ºC in autumn, high precipitations in winter, high percentage of agro-forestry areas and schlerophyllous vegetation, low land slope and low cattle density showed shorter periods of inactive overwintering of *Culicoides* females. The following two models had the same ability to predict the data, being within 2 DIC units of the best model (Annex 12).

Regarding the *C. pulicaris* overwinter period, the best model (DIC= 331.78, annex 13) showed that accumulated degree days over 10ºC in winter had significant negative effect on the overwinter period of this species (Table 10). Therefore, low temperatures during winter season led to an increase of overwintering days of *C. pulicaris* species.

Table 7. Estimated posterior mean values and credible intervals when more than 95% of posterior values are above or below zero for the coefficients for environmental fixed variables of the best Obsoletus complex model within the three different seasonal metrics. (*): indicate significance for environmental variables. ρ= Spearman's rho. Appearance: percentage of the top models in which the significant variables appear.

Obsoletus seasonal metric	Variable	Estimate (95% credible interval)	Correlation	Appearance
Start of season	DDspr	-0.1909* (-0.3690, -0.0099)	ρ= -0.1498	100%
	Elev	0.3689* (0.1187, 0.6109)	ρ= 0.4524	100%
	Pwin	-0.0499 (-0.2636, 0.1642)	ρ= -0.3308	80%
	Cattle	0.0647 (-0.2285, 0.3507)	ρ= -0.3067	60%
	Phapr	-0.4132* (-0.7059, -0.1166)	ρ= -0.2912	50%
	Sum females	-0.3461* (-0.4930, -0.1945)	ρ= -0.4635	100%
End of season	DDsum	0.2525 (-0.3093, 0.7646)	ρ= -0.1672	90%
	Elev	0.0917 (-0.4543, 0.6520)	ρ= -0.3205	60%
	Psum	-0.0276 (-0.5824, 0.4888)	ρ= 0.2557	50%
	(DDsum*Psum)	-0.1625 (-0.5052, 0.1778)	ρ= -0.0811	50%
	Cattle	0.7915* (0.2030, 1.3990)	ρ= 0.3696	40%
	BrdMix	0.3949 (-0.0690, 0.8540)	ρ= 0.2332	10%
	Phsep	-0.0828 (-0.6244, 0.3695)	ρ= 0.1625	60%
	Sum females	-1.0710 (-3.7390, 1.6440)	ρ= 0.2800	100%
Overwinter period	DDaut	-3.687 (-7.715, 0.5369)	ρ= -0.1349	100%
	Paut	-4.356 (-8.996, 0.3213)	ρ= -0.1855	60%
	(DDaut*Paut)	-5.09* (-9.862, -0.3997)	ρ= 0.0623	60%
	Elev	9.8* (4.725, 14.9)	ρ= 0.3860	100%
	NatGras	-4.556 (-9.212, 0.0162)	ρ= 0.0924	80%
	Phsep	-7.653* (-14.64, -2.248)	ρ= -0.2272	30%
	Sum females	-7.098* (-11.06, -3.293)	ρ= -0.4184	100%

Table 8. Estimated posterior mean values and credible intervals when more than 95% of posterior values are above or below zero for the coefficients for environmental fixed variables of the best *C. imicola* model within the three different seasonal metrics. (*): indicate significance for environmental variables. ρ= Spearman's rho. Appearance: percentage of the top models in which the significant variables appear.

***C. imicola* seasonal metric**	**Variable**	**Estimate (95% credible interval)**	**Correlation**	**Appearance**
Start of season	No significant effects detected			
End of season	DDsum	-0.0994 (-0.2851, 0.0957)	ρ= -0.2074	90%
	DDaut	-0.2166 (-0.6704, 0.206)	ρ= 0.2557	60%
	Paut	0.0311 (-0.2218, 0.3149)	ρ= 0.1864	60%
	Elev	-0.4234* (-0.7498, -0.0933)	ρ= -0.5060	100%
	Phsep	-0.2721 (-0.7194, 0.1265)	ρ= 0.1017	100%
	Sum females	0.0981 (-0.0247, 0.2195)	ρ= 0.3076	100%
Overwinter period	DDwin	-2.879 (-11.38, 5.771)	ρ= -0.2748	100%
	Pwin	-0.1633 (-8.911, 8.264)	ρ= 0.0929	70%
	Paut	-1.371 (-13.27, 10.52)	ρ= 0.1319	100%
	(DDwin*Pwin)	6.09* (1.635, 10.61)	ρ= 0.1438	70%
	AgFor	-0.2462 (-8.815, 8.652)	ρ= 0.0649	60%
	NatGras	-1.194 (-7.839, 5.457)	ρ= 0.0906	50%
	Sum females	-5.524 (-11.65, 0.4684)	ρ= -0.2926	100%

Table 9. Estimated posterior mean values and credible intervals when more than 95% of posterior values are above or below zero for the coefficients for environmental fixed variables of the best *C. newsteadi* model within the three different seasonal metrics. (*): indicate significance for environmental variables. ρ= Spearman's rho. Appearance: percentage of the top models in which the significant variables appear.

***C. newsteadi* seasonal metric**	**Variable**	**Estimate (95% credible interval)**	**Correlation**	**Appearance**
Start of season	DDwin	-0.5465* (-0.9463, -0.1563)	ρ= -0.0917	100%
	DDspr	0.1269 (-0.3071, 0.5405)	ρ= -0.2207	40%
	Pspr	0.0578 (-0.2488, 0.4102)	ρ= 0.0310	80%
	(DDspr*Pspr)	-0.1401(-0.3541, 0.0730)	ρ= -0.1465	50%
	Elev	-0.2341 (-0.6747, 0.1988)	ρ= 0.1470	100%
	Sheep	-0.5449* (-0.8769, -0.2207)	ρ= -0.3836	70%
	AgFor	-0.0843 (-0.4123, 0.2446)	ρ= -0.0669	30%
	Sum Females	-0.4168* (-0.6471, -0.1876)	ρ= -0.3607	100%
End of season	Elev	-0.208 (-0.4604, 0.0545)	ρ= -0.2329	80%
	Cattle	-0.2167 (-0.4709, 0.0329)	ρ= -0.1564	60%
	Phsep	-0.123 (-0.6249, 0.3647)	ρ= -0.0045	70%
	Sum females	0.1006 (-0.1068, 0.3035)	ρ= 0.1319	100%
Overwinter period	DDaut	-4.826* (-9.342, -0.326)	ρ= -0.2406	100%
	Pwin	-7.323* (-12.75, -2.167)	ρ= 0.0005	100%
	Paut	-0.2005 (-6.883, 6.307)	ρ= 0.1899	90%
	(DDaut*Paut)	-3.828 (-7.768, 0.05875)	ρ= -0.2991	90%
	Slope	5.829* (1.93, 9.873)	ρ= 0.2521	100%
	Cattle	5.721* (0.3252, 11.26)	ρ= 0.2850	100%
	AgFor	-4.107* (-9.37, -0.899)	ρ= -0.1817	50%
	NatGras	-2.402 (-7.154, 2.121)	ρ= 0.1031	40%
	SchVeg	-4.116* (-7.917, -0.3474)	ρ= -0.2521	50%
	Sum females	-3.728 (-7.423, -0.0946)	ρ= -0.2884	100%

Table 10. Estimated posterior mean values and credible intervals when more than 95% of posterior values are above or below zero for the coefficients for environmental fixed variables of the best *C. pulicaris* model within the three different seasonal metrics. (*): indicate significance for environmental variables. ρ= Spearman's rho. Appearance: percentage of the top models in which the significant variables appear.

***C. pulicaris* seasonal metric**	**Variable**	**Estimate (95% credible interval)**	**Correlation**	**Appearance**
Start of season	DDwin	-0.3234* (-0.6514, -0.05812)	ρ= -0.3921	100%
	Phmarch	0.1949 (-0.08287, 0.4831)	ρ= 0.1218	80%
	Sum females	0.03511 (-0.2028, 0.2844)	ρ= 0.0314	100%
End of season	DDsum	-0.1236 (-0.5477, 0.4025)	ρ= 0.0854	100%
	Psum	-0.4293 (-1.024, 0.1336)	ρ= -0.2488	100%
	(DDsum*Psum)	-0.1615 (-0.5296, 0.1518)	ρ= 0.0937	100%
	Sheep	0.1392 (-0.2747, 0.4722)	ρ= 0.1600	70%
	Phsep	0.4447 (-0.1483, 1.283)	ρ= 0.0572	100%
	Sum females	0.1095 (-0.2652, 0.423)	ρ= 0.0297	100%
Overwinter period	DDwin	-7.69* (-13.49, -1.714)	ρ= -0.3183	40%
	DDaut	-0.5415 (-6.48, 5.446)	ρ= -0.2428	40%
	Paut	1.08 (-3.408, 5.579)	ρ= 0.0909	100%
	(DDaut*Paut)	-3.431 (-8.402, 1.449)	ρ= -0.0826	100%
	Sum females	-2.708 (-6.681, 1.163)	ρ= -0.1891	100%

In summary (Table 11), Obsoletus complex and *C. newsteadi* were the species for which most significant environmental effects on seasonality were detected. Accumulated degree days over 10ºC during winter was the variable with most significant effects among the species (33.3%) with the exception of Obsoletus complex. This variable increased the activity period of *C. newsteadi* and *C. pulicaris* whereas seemed to increase the overwintering period of *C. imicola*. The high *Culicoides* population and low elevation played also an important role among the species (25%) excepting *C. pulicaris*. Sheep densities only affected seasonality of *C. newsteadi*.

Regarding the end of season models, significant environmental effects could only be detected for two species with elevation affecting *C. imicola* and cattle affecting Obsoletus complex species. This is probably linked to the finding that the timing of the end of the adult season was much more variable across species than the timing of the start of the season.

The environmental variables showed different effects on the overwinter periods between studied taxa (Table 11). *Culicoides imicola* overwintering was affected by temperature and precipitation during winter whereas Obsoletus complex was affected by the same metrics but in autumn, in addition to the elevation and the photoperiod in September. For *C. newsteadi*, length of overwinter was affected by six variables including topography, land cover, livestock animals, temperature, and also precipitation. Meanwhile, *C. pulicaris* overwinter was only affected by the temperature in winter.

Table 11. Summary of the significant environmental parameters for each species and seasonal metric. Appearance: Percentage of seasonal metrics in which the variable was significant across all the species. Elev. Elevation; AgFor: Agro-forestry areas; SchVeg: Schlerophyllous vegetation; DDwin/aut/spr: Accumulated degree days over 10ºC in winter, autumn and spring; Paut/win: Precipitation in autumn and spring; Phapr/sep: Photoperiod in April and September; sum fem: *Culicoides* female abundance. (+): positive effect, (-): negative effect.

Species	Seasonal metric	Elev	Slope	Cattle	Sheep	AgFor	SchVeg	DDwin	DDaut	DDspr	Pwin	Paut	Phapr	Phsep	Sum fem
C. imicola	START														
	END	(-)													
	OW							(+)			(+)				
Obsoletus complex	START	(+)								(-)			(-)		(-)
	END			(+)											
	OW	(+)							(-)			(-)		(-)	(-)
C. newsteadi	START				(-)			(-)							(-)
	END														
	OW		(+)	(+)		(-)	(-)		(-)		(-)				
C. pulicaris	START							(-)							
	END														
	OW							(-)							
	Appearance	25%	8.3%	16.7%	8.3%	8.3%	8.3%	33.3%	16.7%	8.3%	16.7%	8.3%	8.3%	8.3%	25%

4.4. Discussion

This is the first time that adult seasonality has been quantified over such a large surveillance system and related to such a comprehensive range of environmental drivers encompassing climate, topography, hosts and landscape. The results obtained have been useful for the knowledge of the seasonal activity of this species in Spain and the environmental requirements for these four vectors species in Spain. These valuable data could help us to understand suitable zones and the environmental circumstances that are favourable for the activity of these insects, which should be taken into account in order to prevent possible BTV outbreaks.

Despite its southerly distribution in Europe, the most important vector species *C. imicola* followed by Obsoletus complex were the most abundant and frequent species collected during the National Surveillance Program which agrees with results recorded by Calvete (2008, 2009a, 2009b), Talavera *et al*. (2015) and Cuéllar *et al*. (2018b) using the same data. The maximum individuals collected of *C. imicola* in Cáceres, *C. newsteadi* in Badajoz, Obsoletus complex in Girona and *C. pulicaris* in Toledo support the results obtained in the previous chapter, where Obsoletus complex were dominant in Northern sites while *C. newsteadi* and *C. imicola* seemed to be more abundant in central and southern parts of mainland Spain.

The length of the adult activity periods was different within the four species by 8 or 12 weeks depending on the site. The temperature, which is a key factor for insects development and activity (Alekseev *et al*. 2007) played an important role in the models and it should be taken into account for the SVPF (EFSA 2017). In fact, the temperature such as the accumulated degree days during winter were an important explanatory variable appearing in a 33.3% of all the species models which demonstrates the importance of the temperature during unfavourable seasons, affecting the development, survival and later seasonal appearance of these insects (Sanders *et al*. 2011, Burgin *et al*. 2013, White *et al*. 2017).

The longest *Culicoides* activity periods were in south sites of Iberian Peninsula and Mediterranean sites due to its high temperatures during the year, for example, *C. imicola* had longer periods in sites with high humidity (Rawlings and Mellor 1994) such as coastal zones (Málaga, Cádiz, Balearic Islands) supporting the study of Conte *et al*. (2007), Talavera *et al*. (2015) and Cuéllar *et al*. (2018b); hence, the transmission season would be longer in this sites since the season of this species overlaps with Palaearctic species.

Sites in the North (Pontevedra and Navarra) had longer periods for Obsoletus complex species while *C. newsteadi* and *C. pulicaris* recorded shorter periods in northern sites such as Lugo and Guadalajara. These results must be considered in order to evaluate the SVFP.

4.4.1. Northern species

Obsoletus complex recorded significant effect of accumulated degree days over 10º in spring and autumn. These findings are consistent with previous studies where Obsoletus complex species had a lower optimum temperature of 14.2 ºC on field (Conte *et al*. 2007). Other studies demonstrated that adults of this species appeared on early dates of the year during low average spring temperatures with seasonal peaks between May and July in Spain and Italy (Miranda *et al*. 2004, Conte *et al*. 2007, Talavera *et al*. 2015), and disappeared late during low mean temperatures in autumn in Portugal (Ramilo *et al*. 2017). Furthermore, despite Obsoletus complex was previously found to be more abundant in arid sites (Conte *et al*. 2007); our results demonstrated that precipitation in autumn reduced the overwinter period of this species. Otherwise, *Culicoides* females abundance had also a positive effect on the abundance of Obsoletus complex as well as the photoperiod. Effects of the latter were non-significant in other studies of the Obsoletus complex species in laboratory conditions (Lühken *et al*. 2015) or even on field data (Searle *et al*. 2014) but not in the present study. These disparities could be related to the lower latitudinal range used in Searle *et al*. (2014) that spanned less variability in photoperiod than the present study.

The topography, namely high elevation, had significant negative effects on adult seasonal activity for many of the species included in this study. In case of Obsoletus complex, elevation played an important role for this species showing longer activity periods in lower elevation sites in contrast to other works in Italy where Obsoletus complex species were favoured by high slope and elevation (Capela *et al*. 2003, Torina *et al*. 2004, Conte *et al*. 2007). These results could be related with the host distribution in Spain since cattle density was another influential factor that increased the length of the activity season. Though some previous studies have shown a preference for Obsoletus complex females to bite sheep (Calvo *et al*. 2012), in other studies Obsoletus complex species have been more generally associated with livestock farms (Talavera *et al*. 2015). In fact, the present work agrees with the results obtained by a recent study conducted by Elbers *et al*. (2018) where high Obsoletus complex biting rates were observed on heifers. In addition, this species breed upon old and fresh manure (Takken *et al*. 2008, González *et al*. 2013b) thus this species can be more abundant in sites with high cattle livestock.

The species with the longest overwinter period in Spain was *C. pulicaris*. This result differed from the long activity periods recorded by Sanders *et al* (2011) for this species in UK. It is a widespread species in Spain with similar distribution of Obsoletus complex, therefore, this species could adapt to a wide range of eco-climatic circumstances (Ortega *et al*. 1998, Ducheyne *et al*. 2013). The models showed unexpected results and, in agreement of the results obtained by Purse *et al*. (2012), the environmental variables did not explain much variance in population parameters for *C. pulicaris*. The adult activity of this species was closely related to temperature (Purse *et al*. 2004a, Sanders *et al*. 2011, Searle *et al*. 2012), therefore, accumulated degree days over 10ºC during winter was the only variable that showed a significant increase in the length of adult activity of *C. pulicaris.*

4.4.2. South-western species

Topography and climate were determinant factors for the abundance and seasonality of *C. imicola* and *C. newsteadi*, which agrees with the results obtained by Acevedo *et al*. (2010) that showed that *C. imicola* was favoured by hot and long spring days in lower altitude sites and extensive plains (also shown by that of Conte *et al*. (2003, 2007) and Torina *et al*. (2004) in Italy). Otherwise, the overwinter period of *C. imicola* was longer in sites with high temperatures and rainfall during winter, probably related with the absence of this species during that season and also because immature stages have preference for breed in dry areas that are not waterlogged (Conte *et al*. 2007, Purse *et al*. 2007, Foxi and Delrio 2010, Ramilo *et al*. 2017). In fact, *C. imicola* was the species that appear as adult latest during the year with seasonal peaks during its optimum temperature between August and October when the land-surface temperature remains high (Ortega *et al*. 1999, Miranda *et al*. 2004, Purse *et al*. 2004a, Talavera *et al*. 2015, Grimaud *et al*. 2018).

Despite *C. newsteadi* has previously demonstrated similar distribution pattern than *C. imicola* (Ducheyne *et al*. 2013, Ramilo *et al*. 2017), the present study has shown that this species were affected by different environmental factors to *C. imicola*. As well as the Obsoletus complex and *C. imicola*; the species *C. newsteadi* was significant affected by the topography. Sites with low land slope showed longer activity periods of this species. Other works showed a decrease of abundance with the altitude, which could be associated with the slope (e. g. of the mountains) (Torina *et al*. 2004, Foxi *et al*. 2016). The temperature during winter and autumn also had significant impact on this species showing high abundance during winter in sites with high minimum temperatures (Ortega *et al*. 1999, Purse *et al*. 2004a). Precipitation was also considered an important factor for *C. newsteadi* (Ducheyne *et al*. 2013), therefore, rainy days during winter was also a significant influence for the phenology of this species, probably because precipitation is also related with the humidity, a variable that affects activity and survival of adult *Culicoides*, protecting them from desiccation (Mellor *et al*. 2000, Wittmann *et al*. 2002, Purse and Rogers 2009).

The short overwinter periods of *C. newsteadi* could be related with its breeding on particular lands being more abundant in agro-forestry and schlerophyllous vegetation areas (Foxi and Delrio 2010, González *et al*. 2013b, Ramilo *et al*. 2007) that are typical vegetation in Mediterranean countries. *Culicoides* female abundance and hosts were also determinant factors for the activity of *C. newsteadi*. This result is supported by prior studies that have found that this species prefers sheep as a host (Garros *et al*. 2011, Calvo *et al*. 2012, Martínez-de la Puente *et al*. 2015, Slama *et al*. 2015). In fact, early stages of this species have been recorded in sheep livestock holdings (Foxi and Delrio 2010, González *et al*. 2013b). However, models showed that sites with high cattle density had shorter adult activity periods for *C. newsteadi* despite some studies recorded that this species also feed upon cattle (Martínez-de la Puente *et al*. 2015). This fact was probably conditioned with the inverse distribution of livestock and presence of *C. newsteadi* in Spain where sites with high cattle density coincide with sites of low abundance of this species.

4.5. Conclusions

This study has demonstrated the eco-climatic and seasonal differences of this four species present in Spain, as well as a contribution to understand the seasonal behaviour and the biology of these species. *Culicoides* abundance, topography, temperature and hosts were elements that mostly affect the seasonal adult activity of these vector insects and will, in turn, determine the SVFP of these species. Hence, areas with suitable environmental circumstances for each particular species should be considered in future surveillance of *Culicoides* species in Spain to detect and prevent possible infections of *Culicoides*-borne viruses.

4.6. Annexes

Annex 1. Summary of the variables used in the literature for the presence (pres.), phenology (phen.), abundance (abund.) and distribution (distr.) of Obsoletus complex (OBS), *C. imicola* (IMI), *C. newsteadi* (NEW) and *C. pulicaris* (PUL). Clim.: Climate, land-cov.: land cover, Phot.: photoperiod, spa. loc.: spatial location, top.: topography.

Source reference	**OBS**	**IMI**	**NEW**	**PUL**	**Variables included**	***Culicoides* association**
Braverman *et al*. (1974)	X				Breeding sites	Pres.
Mellor and Pitzolis (1979)	X	X			Breeding sites	Pres.
Rawlings and Mellor (1994)	X	X			Spa. loc.	Phen.
Ortega *et al* (1998)	X	X		X	-	Phen., distr., abund
Ortega *et al*. (1999)		X	X	X	Clim.	Phen., abund.
Conte *et al*. (2003)		X			Clim.., top.	Pres., distr.
Capela *et al*. (1993)		X			Breeding sites	Abund., distr.
Miranda *et al*. (2004)	X	X			Hosts	Phen., abund.
Tatem *et al*. (2003)		X			Soil	Pres.
Purse *et al*. (2004a)	X	X	X	X	Soil, top.	Pres.
Torina *et al*. (2004)	X	X	X	X	BTV Outbreaks	Pres., distr.
Purse *et al*. (2007)	X	X		X	Clim.	Pres., abund.
Conte *et al*. (2007)	X	X			Clim., top., soil, land-cov.	Abund., distr.
Carpenter *et al*. (2008b)	X			X	Clim.	Phen., abund.
Takken *et al*. (2008)	X			X	Climate, spa. loc.	Abund., distr.
Calvete *et al*. (2009a)	X	X		X	PPT, soil, hosts	Presence
Acevedo *et al*. (2010)		X			Spa.loc., top., Clim., hosts, soil.	
Foxi and Delrio (2010)	X	X	X	X	Breeding sites	Phen., abund.
Garros *et al*. (2011)	X		X	X	Hosts	Blood source
Sanders *et al*. (2011)	X			X	Hosts, top.	Phen., abund.
Calvo *et al*. 2012	X		X	X	Hosts	Blood source
Purse *et al*. (2012)	X			X	Hosts, top., soil	Abund.
Searle *et al*. (2012)				X	Clim., Phot., land-cov.	Phen., abund.

Annex 1. (Continued)

Source reference	**OBS**	**IMI**	**NEW**	**PUL**	**Variables included**	***Culicoides* association**
Ducheyne *et al*. (2013)	X	X	X	X	Clim., soil, top., land-cov.	Abund., distr
González *et al*. (2013a)	X	X	X	X	Hosts, spa. loc.	Phen., abund., distr.
González *et al*. (2013b)	X	X	X	X	Breeding sites	Phen., abund.
Harrup *et al*. (2013)	X			X	Breeding sites	Abund.
Zimmer *et al*. (2013a)	X				Breeding sites	Pres.
Peters *et al*. (2014a)		X			Clim., soil	Phen. Distr.
Peters *et al*. (2014b)		X			soil	Distr.
Zimmer *et al*. (2014a)	X			X	Breeding sites, hosts	Phen., abund.
Zimmer *et al* (2014b)	X			X	Breeding sites	Abund.
Martínez- de la Puente *et al*. (2015)					Hosts	Blood source
Talavera *et al* (2015)	X	X		X	Clim., top., hosts, land-cov.	Phen., abund., distr.
Slama *et al* (2015)		X	X		Hosts	Blood source
Zimmer *et al*. (2015)	X				Hosts	Behaviour
Martínez-de la Puente *et al*. (2017)		X			Hosts	Blood source
Kameke *et al*. (2017)	X			X	Clim., hosts	Phen., abund.
Ramilo *et al*. (2017)	X	X	X	X	Climate, land-cov.	Pres., distr.
Talavera *et al*. (2017b)	X	X	X	X	Hosts	Blood source
Cuéllar *et al*. (2018a)	X	X		X	Clim., soil, land-cov.	Pres.
Cuéllar *et al*. (2018b)	X	X		X	Spa. loc.	Phen., abund., distr.
Elbers *et al*. (2018)	X			X	Hosts	Blood source
Venter *et al*. (2019)		X			Clim.	Abund., pres.

Annex 2. Top 10 models for Obsoletus complex start of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
Obsoletus complex START of season (n = 84)	0	Null model with no covariates (a= -4.34 ± 0.23)	34.13	207.65	26.72
	6	DDspr + Elev + Pwin + Cattle + Phapr + Sum females	28.94	180.93	-
	8	DDspr + Pspr + Elev + Pwin + (DDspr*Pspr) + SchVeg + Phmarch + Sum females	32.81	180.95	0.02
	6	DDspr + Elev + Pwin + SchVeg + Phmarch + Sum females	30.99	181.19	0.26
	8	DDspr + Pspr + Elev + Pwin + (DDspr*Pspr) + Cattle + Phapr + Sum females	29.98	181.43	0.50
	9	DDspr + Pspr + Elev + Pwin + (DDspr*Pspr) + Cattle + Sheep + Phapr + Sum females	30.00	181.86	0.93
	7	DDspr + Elev + Pwin + Cattle + Sheep + Phapr + Sum females	28.55	182.21	1.28
	6	DDspr+ Phapr + Elev + Pspr + Cattle + Sum females	28.541	182.22	1.29
	5	DDspr + Elev + Pwin + Phmarch + Sum females	30.70	182.34	1.41
	6	DDspr+ Phmarch + Elev + Pspr + Cattle + Sum females	30.828	184.72	3.79
	7	DDspr + DDwin + Elev + Pwin + (DDwin*Pwin) + Phmarch + Sum females	29.86	186.29	5.36

Annex 3. Top 10 models for *C. imicola* start of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 20 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
***C. imicola* START of season** (n = 57)	0	No covariates (a= -4.03 ± 0.20)	24.04	128.62	
	6	DDspr + Pwin + AgFor + SchVeg + Phapr + Sum females	19.50	130.17	1.55
	5	DDspr + Pwin + SchVeg + Phapr + Sum females	18.60	130.21	1.59
	6	DDspr + Pwin + Cattle + SchVeg + Phapr + Sum females	18.78	130.63	2.01
	7	DDspr + DDwin + Pwin + (DDwin*Pwin) + Phapr SchVeg + Sum females	29.79	131.18	2.56
	8	DDspr + DDwin + Pwin + (DDwin*Pwin) + AgFor + SchVeg + Phapr + Sum females	21.24	131.31	2.69
	4	DDspr + Pwin + Phapr + Sum females	16.28	131.78	3.16
	6	DDspr + Pwin + Pspr + SchVeg + Phapr + Sum females	19.21	132.09	3.47
	5	DDspr + Pwin + Cattle + Phapr + Sum females	16.61	132.10	3.48
	6	DDspr + Pwin + Cattle + AgFor + Phapr + Sum females	17.64	132.60	3.98
	8	DDspr + DDwin + Pwin + (DDwin*Pwin) + Cattle + SchVeg + Phapr + Sum females	20.99	132.66	4.04

Annex 4. Top 10 models for *C. newsteadi* start of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. ∆ DIC: Difference with the best model. n= Number of observations with > 5 individuals per trap.

Model	N pred.	Variables	pD	DIC	∆ DIC
C. newsteadi **START of season** (n = 58)	0	No covariates (a= -4.23 ± 0.23)	24.17	134.01	13.67
	8	DDwin + DDspr + Pspr + (DDspr*Pspr) + Elev + Sheep + AgFor + Sum females	26.95	120.34	-
	9	DDwin + DDspr + Pwin + Pspr + (DDspr*Pspr) + Elev + Slope + Sheep + Sum females	27.92	122.29	1.95
	5	DDwin + Elev + Sheep + SchVeg + Sum females	21.57	123.02	2.68
	6	DDwin + Pspr + Elev + Sheep + SchVeg + Sum females	22.56	124.33	3.99
	6	DDwin + Elev + Pspr + (DDspr*Pspr) + SchVeg + Sum females	29.41	124.51	4.17
	5	DDwin + Elev + Sheep + AgFor + Sum females	21.64	124.77	4.43
	8	DDwin + Pwin + Pspr + Elev + Slope + Sheep + SchVeg + Sum females	24.94	124.90	4.56
	6	DDwin + Pspr + Elev + Sheep + AgFor + Sum females	23.01	125.20	4.86
	8	DDwin + DDspr + Pwin + Pspr + (DDspr*Pspr) + Elev + Slope + Sum females	29.87	125.48	5.14
	9	DDwin + DDspr + Elev + Slope + Pwin + Pspr + (DDwin*Pwin) + (DDspr*Pspr) + Sum females	29..48	126.00	5.66

Annex 5. Top 10 models for *C. pulicaris* start of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
***C. pulicaris* START of season** (n = 40)	0	No covariates (a= -4.25 ± 0.30)	8.06	91.12	2.35
	3	DDwin + Phmarch + Sum females	9.85	88.77	-
	4	DDwin + Phmarch + Pwin + Sum females	10.99	89.46	0.69
	2	DDwin + Sum females	8.71	89.48	0.71
	4	DDwin + Phmarch + Elev + Sum females	10.94	89.72	0.95
	4	DDwin + Phmarch + BrdMix + Sum females	10.89	89.88	1.11
	4	DDwin + Phmarch + Sheep + Sum females	11.12	90.25	1.48
	4	DDwin + Phmarch + NatGras + sum females	10.91	90.32	1.55
	3	DDwin + Phapr + Sum females	10.02	90.41	1.64
	5	DDwin + Phmarch + Pwin + PastGras + sum females	12.04	90.51	1.74
	5	DDwin + Pwin + (DDwin*Pwin) + Phmarch + Sum females	11.91	91.00	2.23

Annex 6. Top 10 models for Obsoletus complex end of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. ∆ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	∆ DIC
Obsoletus complex END of season (n = 84)	0	No covariates (a= -1.51 ± 0.33)	32.96	216.39	36.24
	8	DDsum + Elev + Psum + (DDsum*Psum) + Cattle + BrdMix + Phsep + Sum females	22.34	180.15	-
	4	DDsum + DDaut + Phnov + Sum females	21.81	180.16	0.01
	3	DDaut + Phnov + Sum females	19.52	181.06	0.91
	6	DDsum + Elev + Psum + (DDsum*Psum) + Phnov + Sum females	23.74	182.10	1.95
	7	DDsum + Elev + Psum + (DDsum*Psum) + Cattle + Phsep + sum females	30.43	189.17	9.02
	8	DDsum + Elev + Psum + (DDsum*Psum) + Cattle + Sheep + Phnov + Sum females	34.01	194.45	14.30
	4	DDsum + DDaut + Phsep + Sum females	31.50	194.64	14.49
	4	DDsum + Elev + Phsep + Sum females	32.86	195.09	14.94
	4	DDsum + Slope + Phsep + sum females	35.55	195.43	15.28
	8	DDsum + Psum + (DDsum*Psum) + Elev + Cattle + Sheep + Phsep + Sum females	35.80	195.77	15.62

Annex 7. Top 10 models for *C. imicola* end of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. ∆ DIC: Difference with the best model. n= Number of observations with > 20 individuals per trap.

Model	N pred.	Variables	pD	DIC	∆ DIC
C. imicola **END of season** (n =57)	0	No covariates (a= -1.11 ± 0.50)	19.81	63.52	17.84
	6	DDsum + DDaut + Paut + Elev + Phsep + Sum females	23.68	45.68	-
	7	Phsep + Elev + Paut + Cattle + AgFor + NatGras + Sum females	26.48	46.27	0.59
	5	DDaut + DDsum + Phsep + Elev + Sum females	22.18	46.31	0.63
	7	DDaut + DDsum + Phsep + Elev + AgFor + NatGras + Sum females	23.42	46.82	1.14
	7	DDaut + DDsum + Phsep + Elev + Paut + Cattle + Sum females	24.14	46.87	1.19
	7	DDsum + Phsep + Elev + Paut + Cattle + AgFor + Sum females	24.34	47.11	1.43
	6	DDsum + Phsep + Elev + Paut + Cattle + Sum females	23.05	47.50	1.82
	8	DDaut + DDsum + Phsep + Elev + AgFor + NatGras + SchVeg + Sum females	24.11	47.53	1.85
	7	DDaut + DDsum + Phsep + Elev + Paut + (DDaut*Paut) + Sum females	24.06	47.67	1.99
	6	DDsum + Phsep + Elev + Paut + Sheep + Sum females	22.55	47.78	2.10

Annex 8. Top 10 models for *C. newsteadi* end of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 5 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
***C. newsteadi* END of season** (n = 58)	0	No covariates (a= -1.72 ± 0.60)	8.71	130.45	3.64
	4	Elev + Cattle + Phsep + Sum females	13.52	126.81	-
	7	DDaut + Paut + (DDaut*Paut) + Elev + Cattle + Phsep + Sum females	15.76	127.05	0.24
	8	DDaut + Elev + Paut + (DDaut*Paut) + Cattle + AgFor + Phsep + Sum females	16.65	128.20	1.39
	3	DDaut + Phsep + Sum females	13.04	128.73	1.92
	3	DDsum + Phsep + Sum females	13.37	128.77	1.96
	4	DDaut + Elev + Phnov + Sum females	13.05	129.03	2.22
	8	DDaut + Elev + Paut + (DDaut*Paut) + Cattle + SchVeg + Phnov + Sum females	16.70	129.13	2.32
	6	DDaut + Elev + Paut + (DDaut*Paut) + Phnov + Sum females	14.68	129.15	2.34
	8	DDaut + Elev + Paut + (DDaut*Paut) + Cattle + NatGras + Phsep + Sum females	16.64	129.19	2.38
	8	DDaut + Elev + Paut + (DDaut*Paut) + Cattle + Sheep + Phsep + Sum females	16.73	129.24	2.43

Annex 9. Top 10 models for *C. pulicaris* end of the season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
***C. pulicaris* END of season** (n = 40)	0	No covariates (a= -2.22 ± 0.49)	17.50	90.50	22.17
	6	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + Sum females	24.34	68.33	-
	7	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + NatGras + Sum females	25.02	69.88	1.55
	7	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + PastGras + Sum females	24.00	72.00	3.67
	7	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + BrdMix + Sum females	23.30	72.32	3.99
	5	DDsum + Phsep + Psum + (DDsum*Psum) + Sum females	24.18	73.00	4.67
	7	DDsum + Phsep + Psum + (DDsum*Psum) + Cattle + Sheep + Sum females	24.35	73.26	4.93
	6	DDsum + Phsep + Psum + (DDsum*Psum) + (DDaut*Paut) + Sum females	24.74	73.37	5.04
	8	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + PastGras + BrdMix + Sum females	23.39	74.62	6.29
	8	DDsum + Phsep + Psum + (DDsum*Psum) + Sheep + NatGras + PastGras + Sum females	21.85	74.86	6.53
	7	DDsum + DDaut + Psum + Paut + (DDaut*Paut) + Phsep + Sum females	23.17	76.61	8.28

Annex 10. Top 10 models for Obsoletus complex overwinter (OW) season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
Obsoletus complex OW season (n = 38)	0	No covariates (a= 25.29 ± 4.52)	24.95	359.87	15.69
	7	DDaut + Paut + (DDaut*Paut) + Elev + NatGras + Phsep + Sum females	25.62	344.18	-
	8	DDaut + Paut + (DDaut*Paut) + Elev + Cattle + NatGras + Phsep + Sum females	26.39	344.54	0.36
	8	DDaut + Paut + (DDaut*Paut) + Elev + NatGras + SchVeg + Phnov + Sum females	25.26	345.71	1.53
	8	DDaut + Paut + (DDaut*Paut) + Elev + Cattle + NatGras + SchVeg + Sum females	27.78	346.35	2.17
	7	DDaut + Paut + (DDaut*Paut) + Elev + SchVeg + Phsep + Sum females	26.39	346.40	2.22
	7	DDaut + Paut + (DDaut*Paut) + Elev + SchVeg + Phnov + Sum females	23.95	348.71	4.53
	5	DDaut + Elev + NatGras + Phnov + Sum females	21.84	351.65	7.47
	6	DDaut + Elev + NatGras + SchVeg + Phnov + Sum females	21.99	351.75	7.57
	7	DDaut + Elev + Cattle + NatGras + SchVeg + Phnov + Sum females	22.31	352.71	8.53
	6	DDaut + Elev + Cattle + NatGras + Phnov + Sum females	22.26	352.81	8.63

Annex 11. Top 10 models for *C. imicola* overwinter (OW) season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 20 individuals per trap.

Model	**N pred.**	**Variables**	**pD**	**DIC**	**Δ DIC**
C. imicola **OW season** (n = 29)	0	No covariates (a= 21.33 ± 4.67)	7.41	223.32	51.52
	7	DDwin + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Sum females	16.80	171.80	-
	8	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Sum females	16.40	175.22	3.42
	7	DDwin + DDaut + Paut + AgFor + NatGras + SchVeg + Sum females	17.53	175.59	3.79
	7	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + Sum females	16.32	175.74	3.94
	7	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + NatGras + Sum females	16.09	176.06	4.26
	6	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + Sum females	15.49	176.20	4.46
	6	DDwin + DDaut + Paut + NatGras + SchVeg + Sum females	16.62	177.15	5.35
	7	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + Sheep + Sum females	15.52	178.81	7.01
	8	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + SchVeg + Sum females	15.87	180.51	8.71
	6	DDwin + DDaut + Paut + AgFor + SchVeg + Sum females	16.17	181.69	9.89

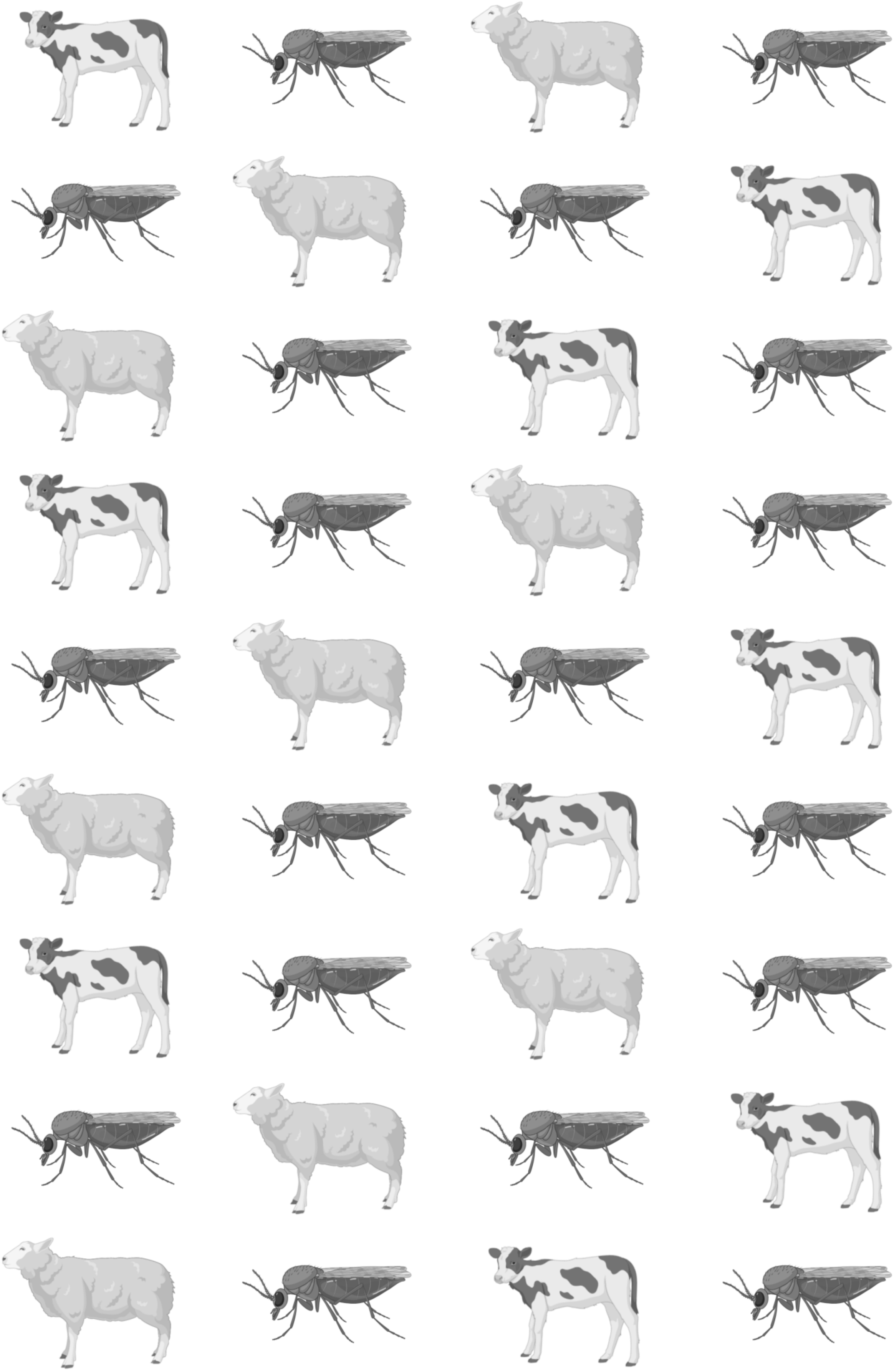
Annex 12. Top 10 models for *C. newsteadi* overwinter (OW) season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 5 individuals per trap.

Model	**N pred.**	**Variables**	**pD**	**DIC**	**Δ DIC**
C. newsteadi **OW season** (n = 35)	0	No covariates (a= -26.27 ± 3.93)	12.06	352.97	17.32
	10	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + AgFor + NatGras + SchVeg + Sum females	17.93	335.65	-
	9	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + AgFor + SchVeg + Sum females	16.79	335.84	0.19
	7	DDaut + Pwin + Slope + Cattle + AgFor + SchVeg + Sum females	14.40	336.72	1.07
	9	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + AgFor + NatGras + Sum females	18.89	337.74	2.09
	8	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + AgFor + Sum females	17.52	337.99	2.34
	8	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + SchVeg + Sum females	18.28	338.75	3.10
	8	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + NatGras + Sum females	19.49	338.79	3.14
	9	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + NatGras+ SchVeg + Sum females	19.62	338.83	3.18
	7	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + Sum females	17.951	338.87	3.22
	8	DDaut + Pwin + Paut + (DDaut*Paut) + Slope + Cattle + Sheep + Sum females	18.10	338.91	3.26

Annex 13. Top 10 models for *C. pulicaris* overwinter (OW) season (best model in grey). a= intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of observations with > 1 individuals per trap.

Model	N pred.	Variables	pD	DIC	Δ DIC
***C. pulicaris* OW season** (n = 35)	0	No covariates (a= 29.51 ± 7.32)	8.73	338.99	7.21
	5	DDwin + DDaut + Paut + (DDaut*Paut) + Sum females	12.54	331.78	-
	6	DDwin + DDaut + Paut + (DDaut*Paut) + PastGras + Sum females	13.48	333.80	2.02
	6	DDwin + DDaut + Paut + (DDaut*Paut) + NatGras + Sum females	13.58	333.99	2.21
	6	DDwin + DDaut + Paut + (DDaut*Paut) + BrdMix + Sum females	13.49	334.12	2.34
	4	DDaut + Paut + (DDaut*Paut) + Sum females	12.24	338.97	7.19
	5	DDaut + Paut + (DDaut*Paut) + BrdMix + Sum females	13.30	339.09	7.31
	5	DDaut + Paut + (DDaut*Paut) + Sheep + Sum females	13.50	339.10	7.32
	5	DDaut + Paut + (DDaut*Paut) + PastGras + Sum females	13.27	340.46	8.68
	5	DDaut + Paut + (DDaut*Paut) + NatGras + Sum females	13.15	340.73	8.95
	5	DDaut + Paut + (DDaut*Paut) + Cattle + Sum females	13.22	340.87	9.09

5. The use of Path Analysis as a model to determine the effects of the environmental factors on the adult seasonality of *Culicoides* species in Spain



5. The use of Path Analysis as a model to determine the effects of the environmental factors on the adult seasonality of *Culicoides* species in Spain

5.1. Introduction

A technique called Path analysis (PA) is frequently used when we want to see the effects of predictor variables on a number of different dependent variables. This technique is easier to understand and has replaced other sophisticated methods such as mathematical models or computer algorithms. In fact, this analysis allows the use of diagrams in order to observe the relationship with the variables (Norman and Streiner 2003).

As seen in the previous chapter (especially Annex 4.6), environmental factors have wide ranging impacts on *Culicoides* populations, including on the presence, abundance and seasonality of adults, aside from the impacts on development and survival and breeding site availability. Therefore, understanding the underlying mechanisms and drivers of these interactions is of great importance for surveillance of *Culicoides* populations and defining the period of risk of arboviruses transmission (Sanders *et al*. 2011). Actually, the environment has important direct and indirect effects on *Culicoides* abundance and seasonality whilst the *Culicoides* female abundance plays an important role as a reservoir in breeding sites, since the reproduction of the *Culicoides* females maintain the population year-round through their oviposition on breeding sites. Cold winters or heavy rain, for example, exert a direct effect on the adult activity period of these insects (Rawlings *et al*. 1998, Alekseev *et al*. 2007), and also an indirect effect via females abundance. In fact, the previous chapter demonstrated that *Culicoides* females abundance had an important impact on the seasonality of these insects. Path analysis was also used in other studies with red deer in Norway (Mysterud *et al*. 2008) and insects like ants in Argentina (Fergnani *et al*. 2008) as a more likelihood way to interpret the relationship between abundance and environmental drivers. Hence, the use of PA allows us to figure out and visualize these kinds of interactions.

In this chapter, we use for the first time PA to link changes in seasonal environmental variables and variation in phenology of adult females of *Culicoides* populations with the seasonal appearance, disappearance and overwinter period of the nulliparous and parous adults of the four different species of *Culicoides* used in the previous chapters (Obsoletus complex, *C. imicola*, *C. newsteadi* and *C. pulicaris*) in order to understand how the environment could affect these species, contrasting between the northern species Obsoletus complex/ *C. pulicaris* and the south-eastern species *C. imicola*/ *C. newsteadi*.

5.2. Material and Methods

To explore the direct and indirect effects of environmental factors and *Culicoides* females abundance on the seasonal metrics in Spain (start of the season, end of the season and length of overwinter) we implemented hierarchical Bayesian structural equation modelling using data from 329 *Culicoides* sampling sites over the period 2005–2010 from the previous chapter obtained during the National Entomosurveillance Program for BTV sponsored by the Spanish Ministry of Rural and Marine Environment. We used hierarchical structural equation models within the Bayesian framework. This modelling is considered suitable for this kind of analysis because it allows a series of hypothesized cause and effect relationships to be captured within a single model, estimating the magnitude of both direct (seasonal metric) and indirect (via *Culicoides* females abundance effects of the independent variables on dependent variables (Shipley 2016). The independent variables were the same used in the previous chapter: climate, land cover, topography, hosts, photoperiod and *Culicoides* females abundance (Table 5) obtained from different sources such as CORINE, SRTM, ENSEMBLES and Robinson *et al*. (2012).

Structural equation modelling was used to examine links between variation in the seasonal metric of *Culicoides* NF and PF, *Culicoides* female abundances and environment variables. Based on our understanding of the system, we developed a PA model for how environmental variables and females abundance directly and indirectly affect the seasonal metric of NF and PF for each four analysed species (*C. imicola*, Obsoletus complex, *C. newsteadi* and *C. pulicaris*) incorporating knowledge on drivers of these individual species from literature. The models quantified the direct effects of climate, land cover, hosts, topography and photoperiod on the seasonal metrics, and the indirect effects of all of these variables on the seasonal metrics via *Culicoides* female abundance (Fig. 23). We did not look for indirect effects of photoperiod through females abundance because we considered that availability of daylight hours may only cause an effect on the phenology of *Culicoides* species through an overcoming of insect diapause (Tauber and Tauber 1976, Isaev 1985). In fact, some significant effects on variation in phenology of *Culicoides* across sites were found in previous studies using surveillance data such as Searle *et al*. (2012, 2014) while in another laboratory study this variable was not significant (Lühken *et al*. 2015).

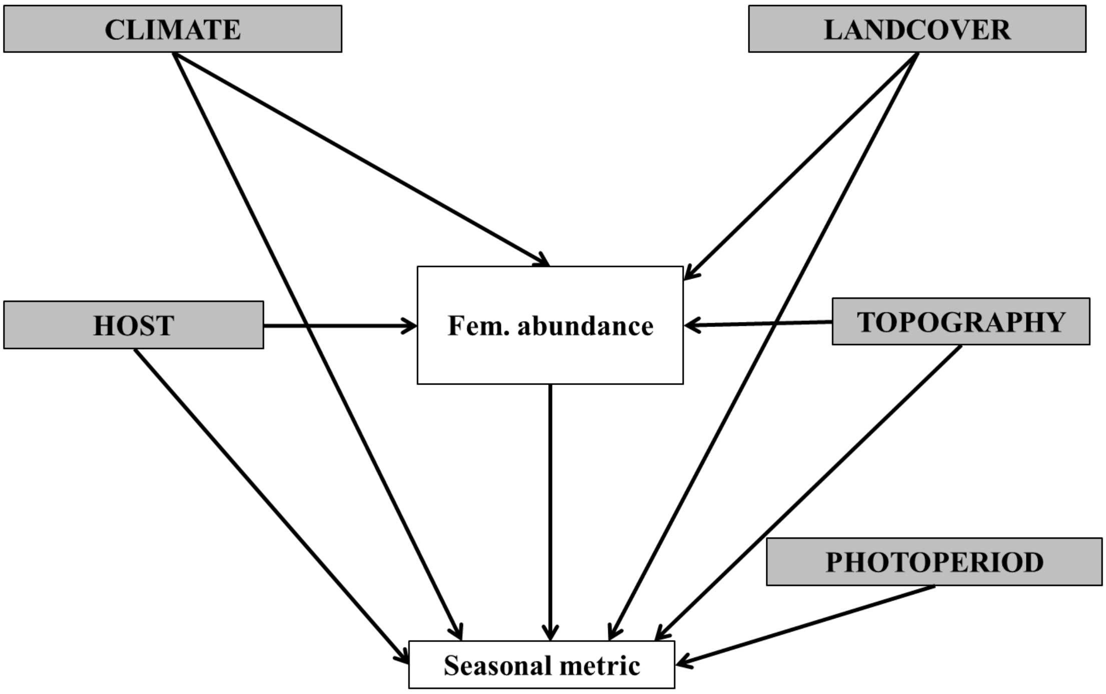


Figure 23. Structural equation model diagram for how the seasonal metrics of different *Culicoides* species are affected directly by environmental variables and indirectly via annual *Culicoides* abundance of females for sites in Spain.

We then fitted the model to site-year observations of phenology spread over five years (2005-2010) from the four species (see Chapter 4, Table 6 for sample sizes per species across sites and years). All models were fitted using WinBUGS (Spiegelhalter *et al*. 2004) software and a Markov chain Monte-Carlo (MCMC) procedure for each model run for 10,000 iterations after an initial burn in of 10,000 iterations to ensure convergence of all model parameters.

5.3. Results

5.3.1. *Culicoides imicola*

5.3.1.1. Seasonal Start PA

Schlerophyllous vegetation, which is a common type of vegetation adapted for Mediterranean hot and dry summers, showed a strong positive effect on *C. imicola* abundance for NF (more than the 95% of credible intervals for the fixed effects greater than zero) (Fig. 24). Sites having higher percentage of Schlerophyllous vegetation showed higher NF abundance, therefore an earlier start of *Culicoides* adult season. The best model for PF was the null model which contained no environmental variables; although, the second best model including six variables had similar support in the data (Δ DIC < 2, Annex 17). However, from these results for PF, we must conclude that our analysis was unable to detect any significant relationships between seasonality and the measured environment variables for *C. imicola*.

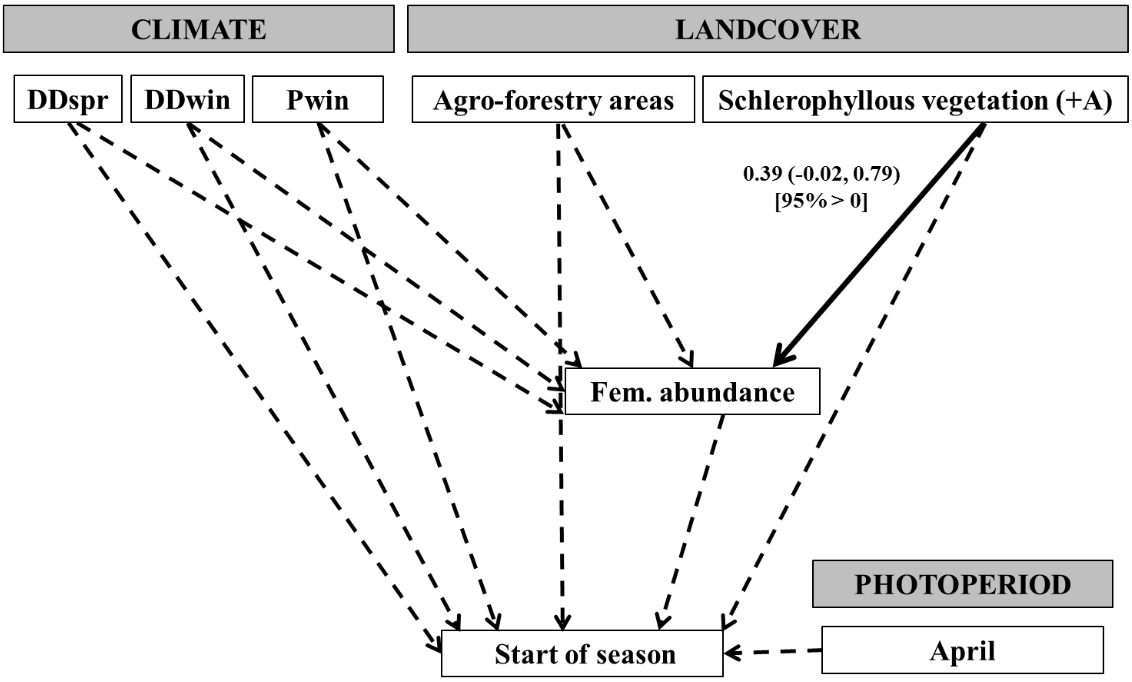


Figure 24. Path diagrams for models for the timing of the start of the season of *C. imicola* nulliparous females (NF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the start of the season and the predictor variable. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.1.2. Seasonal End PA

The models showed that two variables, accumulated degree days over 10ºC in summer and the elevation had strong negative effects on the end of the *C. imicola* NF and PF season (Fig. 25, A and B). In addition, cattle showed a weak effect on the end of season for NF (more than the 90% of credible interval was greater than zero) (Fig. 25, A) and natural grasslands showed a weak positive effect on the end of season for PF (Fig 25, B). Therefore, sites with lower temperatures in summer time, lower elevations, and high percentage of natural grassland areas for PF showed a longer end of season period and, therefore, a longer period of risk of transmission of BTV in autumn.

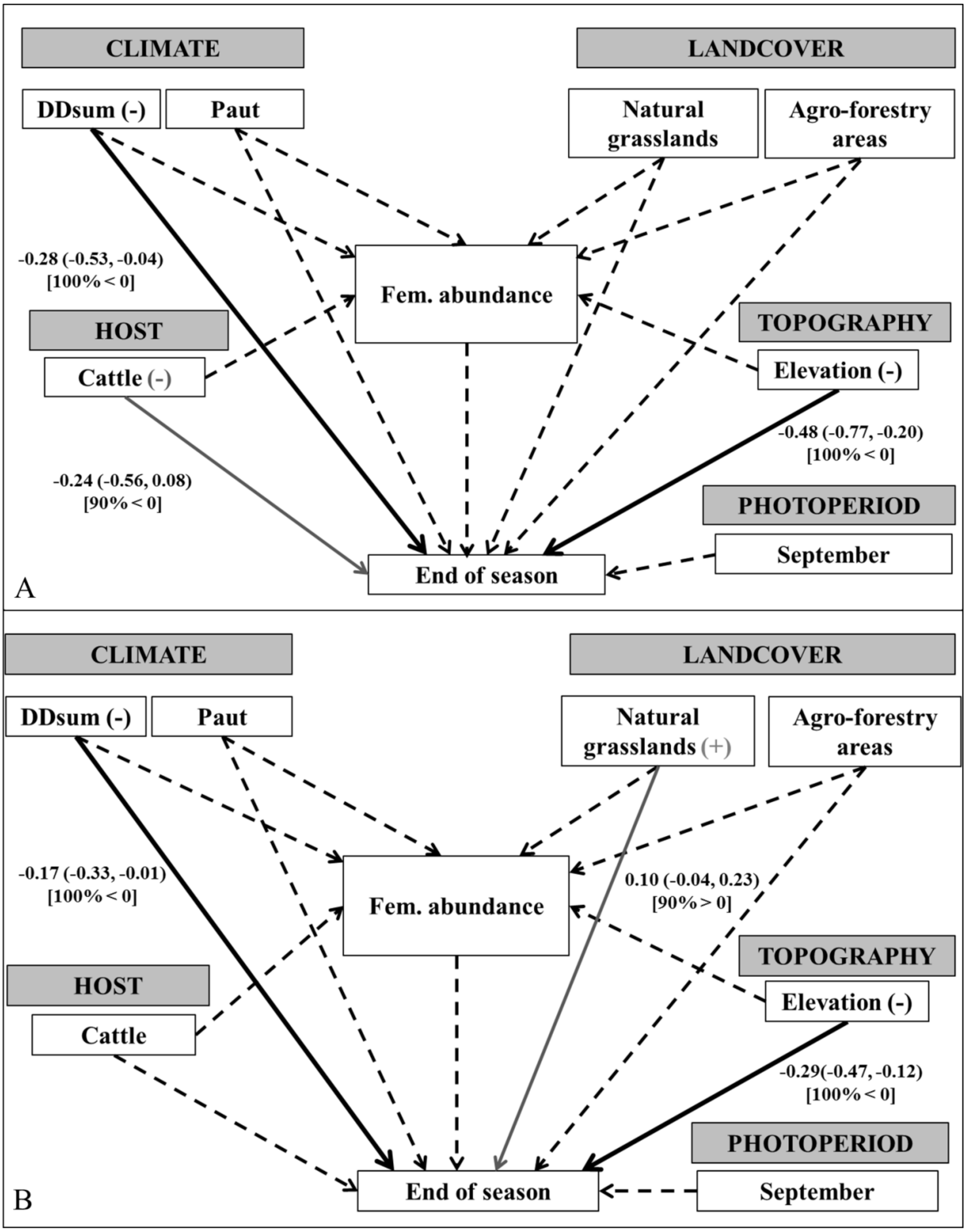


Figure 25. Path diagrams for models for the timing of the end of the season of *C. imicola*. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the end of the season and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.1.3. Overwintering PA

The PA showed that accumulated degree days over 10ºC plus precipitation in winter had a strong positive effect on the length of overwinter of both NF and PF. On the other hand, accumulated degree days over 10º in autumn showed a weak negative effect on the length of overwinter of NF (Fig. 26, A) and precipitation during winter in addition with the *Culicoides* females abundance had a weak negative effect on the length of overwinter of PF (Fig. 26, B). Therefore, sites having low temperatures combined with low precipitation in winter and low number of days over 10ºC in autumn had shorter overwintering periods of NF. In addition, sites with high precipitation in winter and high abundance of *Culicoides* females showed shorter overwintering period of *C. imicola* PF, therefore, longer period of risk of BTV transmission.

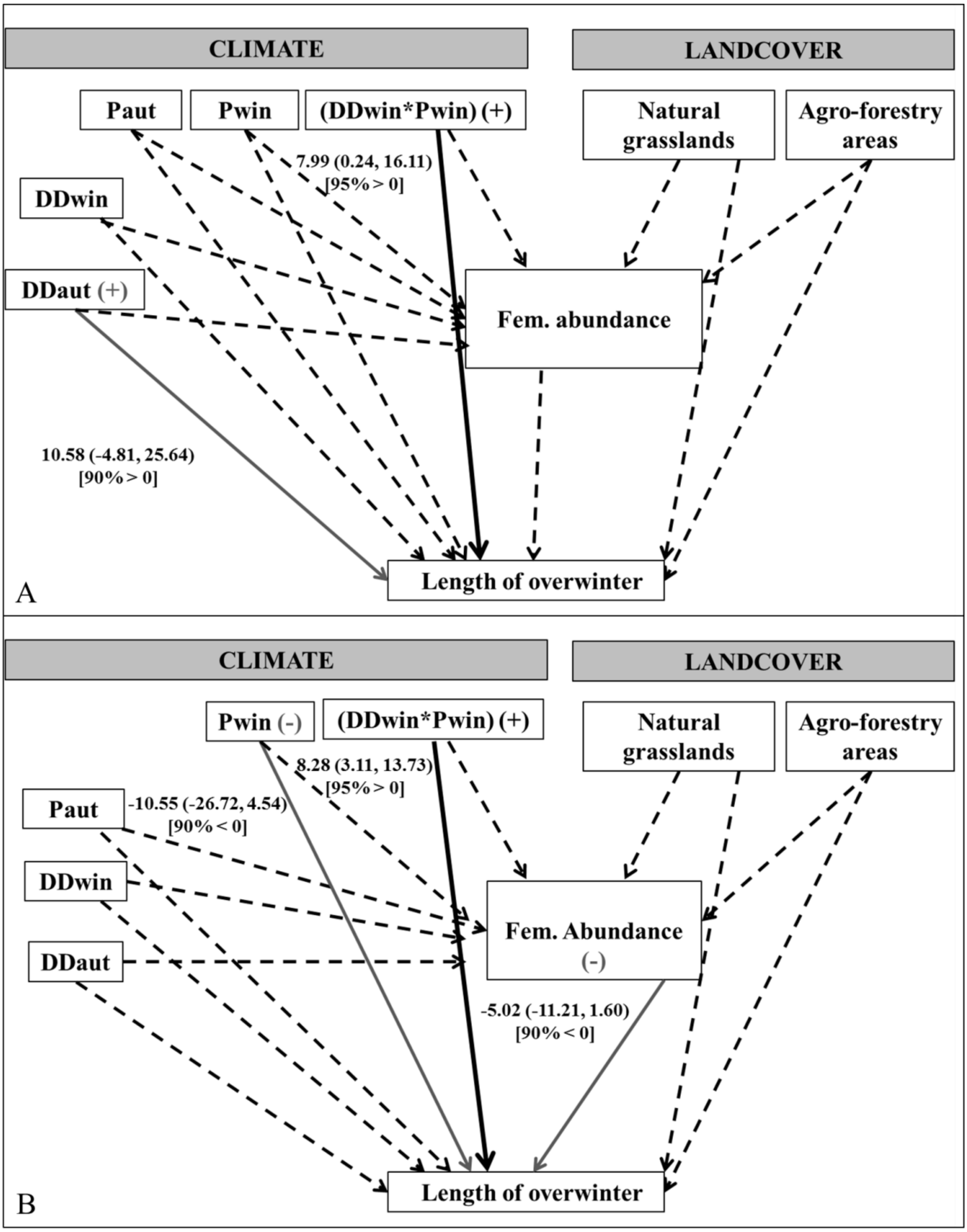


Figure 26. Path diagrams for models for the timing of the length of overwinter period of *C. imicola*. (A): nulliparous females (NF); (B): parous females (PF).Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the length of overwinter and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.2. Obsoletus complex

5.3.2.1. Seasonal Start PA

Female abundance showed a strong effect on the start of the season for both NF and PF; accumulated degree days over 10ºC in spring showed a strong negative effect on the start of the season for NF (Fig. 27, A) and a weak negative effect on the start of the season for PF (Fig. 27, B). The precipitation in spring and cattle livestock showed a weak negative effect on the start of the season for NF. The elevation and photoperiod in April showed a strong positive and negative effect respectively on the start of the season of Obsoletus complex PF, in addition, cattle density showed a weak positive effect on the start of the season of those PF (Fig. 27, B). These results mean that the *Culicoides* season for both NF and PF started earlier in sites with higher Obsoletus complex population and high number of consecutive days over 10ºC in spring. For NF, sites with higher precipitation in spring and higher cattle density had an earlier start of Obsoletus complex NF season. Meanwhile, for PF, sites with lower elevation, higher number of daylight hours in April and lower density of cattle showed a more prompt start of the Obsoletus complex PF season, therefore an earlier period of risk of BTV transmission.

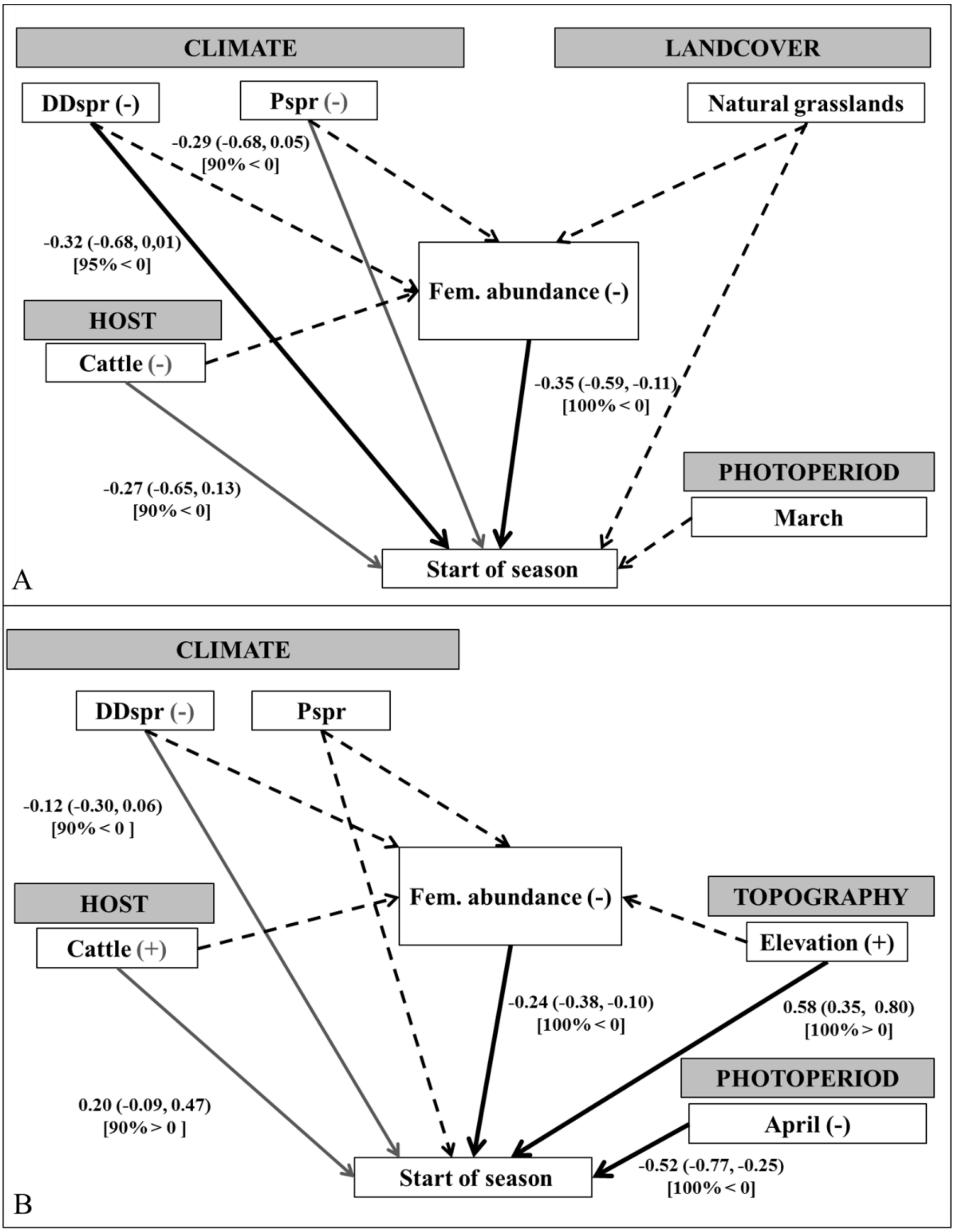


Figure 27. Path diagrams for models for the timing of the start of the season of Obsoletus complex. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the start of season and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.2.2. Seasonal End PA

Cattle abundance and *Culicoides* females abundance had a strong positive effect on the end of the season for both NF and PF (Fig. 28, A and B). Sites having higher density of cattle and Obsoletus complex females population, showed a longer end of Obsoletus complex adult period. Regarding the PF, the slope and the schlerophyllous vegetation showed a strong positive and negative effect respectively on the end of the season (Fig. 28, B). Finally, accumulated degree days over 10ºC in autumn showed a weak positive effect on the end of the season of PF. Overall, sites with schlerophyllous vegetation areas had shorter seasons of PF while sites with high temperatures in autumn in high slope areas showed longer end of season periods, extending the risk of transmission of BTV to later through the year.

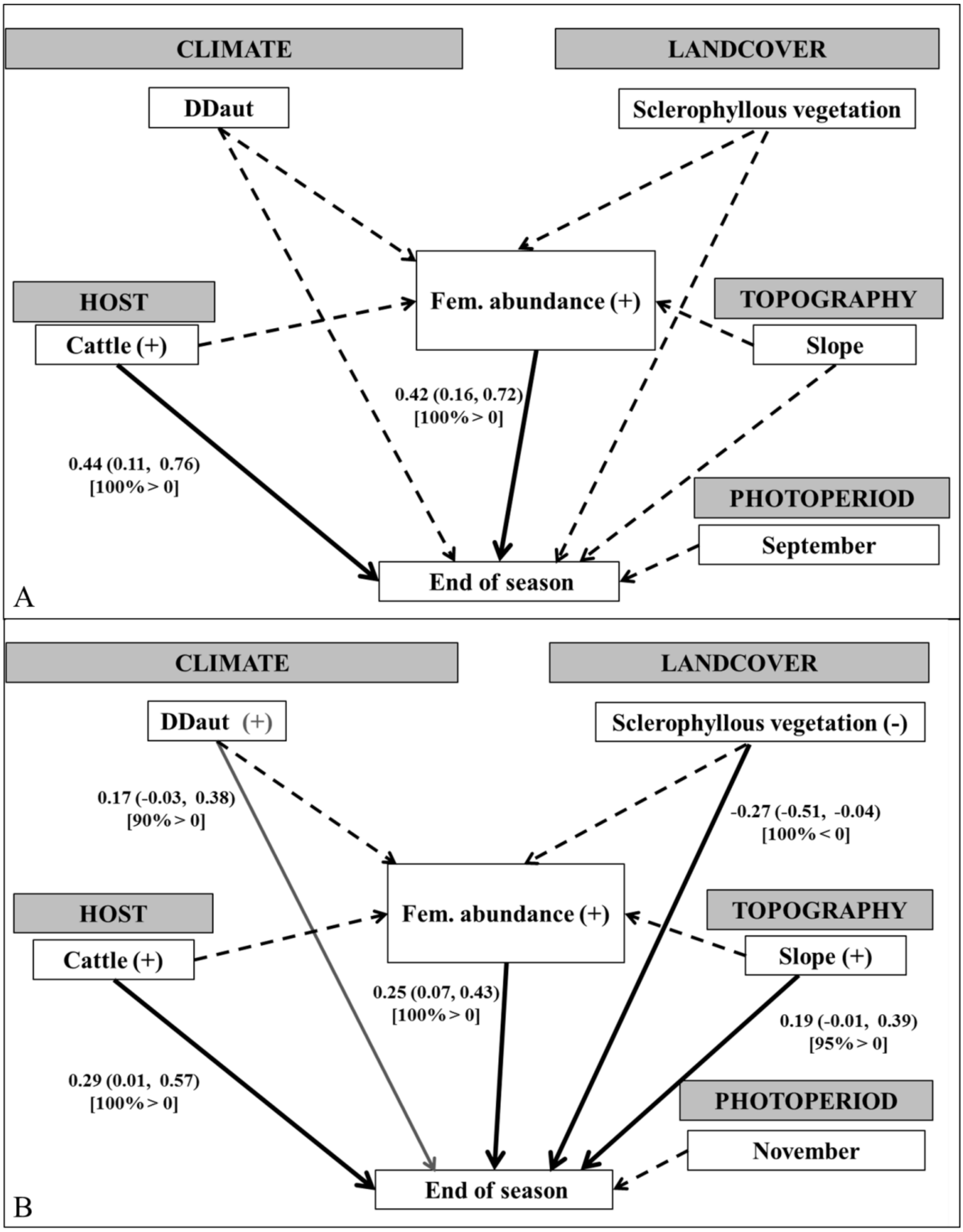


Figure 28. Path diagrams for models for the timing of the end of the season of Obsoletus complex. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the end of season and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.2.3. Overwintering PA

Elevation and the photoperiod in November had a significant positive effect on the length of overwinter of Obsoletus complex NF (Fig. 29) meaning that the overwinter period of NF was longer in sites with higher number of daylight hours during November and in elevated sites. For PF, the best model did not include any variables (the null model); however, the following top model which included seven variables received similar support in the data (Δ DIC < 2, Annex 26). From these results, we must conclude that our analysis for PF was unable to detect any significant relationships between the measured environment variables and seasonality.

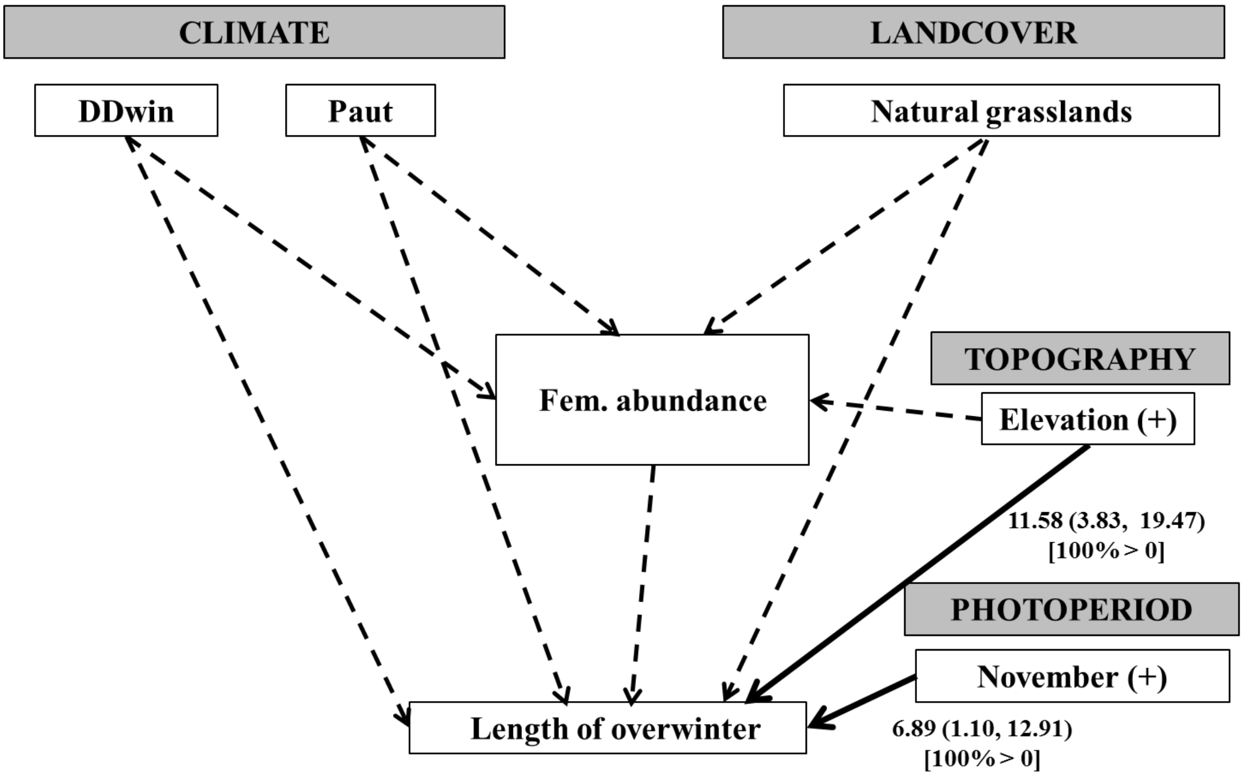


Figure 29. Path diagrams for models for the timing of the length of overwinter period of Obsoletus complex nulliparous females (NF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the length of overwinter and the predictor variable. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.3. *Culicoides newsteadi*

5.3.3.1. Seasonal Start PA

Female abundance showed a strong negative effect on the start of the season of PF (Fig. 30). Accumulated degree days over 10ºC on winter and sheep livestock had a weak negative and positive effect respectively on the start of season of *C. newsteadi* PF. On the other hand, precipitation during winter showed a weak negative indirect effect on the season through females abundance. Therefore, sites with higher average female population, higher number of days over 10ºC in winter season and lower density of sheep showed an earlier start of *C. newsteadi* PF season. In addition, sites with higher precipitation in winter were associated with a decrease in the abundance of *C. newsteadi* females causing an indirect effect on the seasonality of this species. No environmental variables were included in *C. newsteadi* NF best path model.

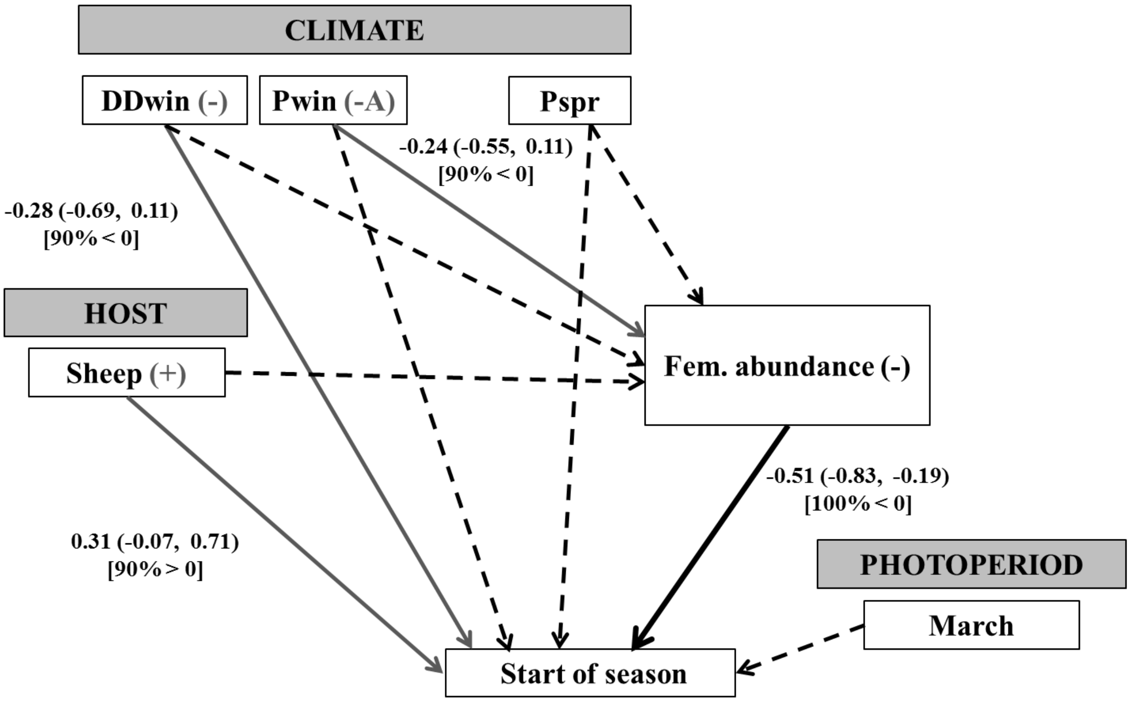


Figure 30. Path diagrams for models for the timing of the start of the season of *C. newsteadi* parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the start of the season and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.3.2. Seasonal End PA

The best path models for the end of season of *C. newsteadi* NF and PF did not include any variables (null model). For PF, the second best model included five variables and received similar support in the data (Δ DIC < 2, Annex 23). However, we must conclude from these results that our analysis was unable to detect any meaningful associations between seasonality and the measured environmental variables for this species.

5.3.3.3 Overwintering PA

Female abundance had a strong negative effect on the *C. newsteadi* NF length of overwinter season (Fig. 32, A) and a weak negative effect on the length of overwinter of PF (Fig. 31, B). In addition, schlerophyllous vegetation and slope had a weak negative and positive effect respectively on the length of overwinter period of NF (Fig. 31, A). Therefore, high female abundance was associated with a decrease in the overwintering period of both NF and PF lengthening the potential BTV transmission period. Meanwhile, sites with higher schlerophyllous vegetation areas and lower slope showed a longer period of activity of *C. newsteadi* NF.

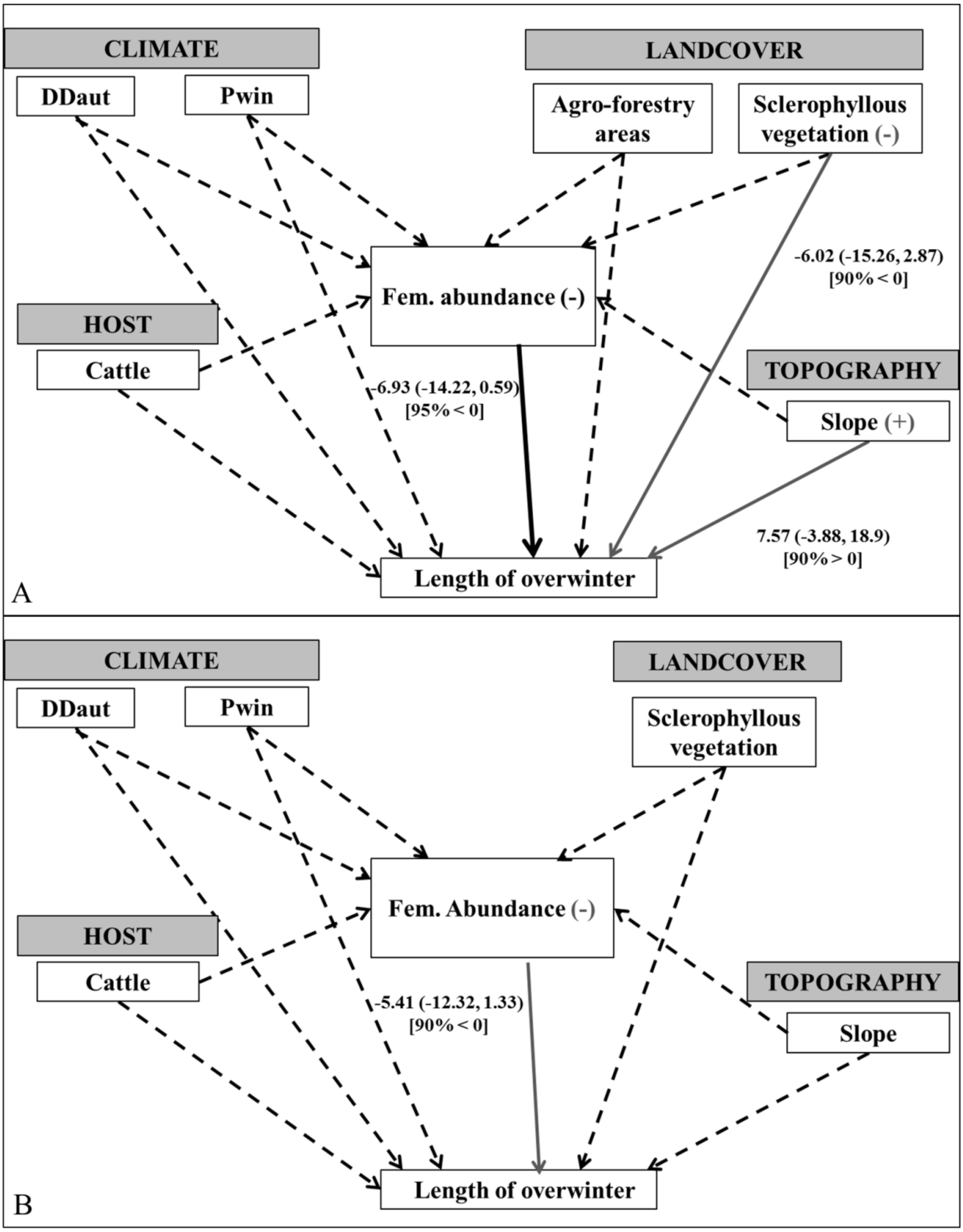


Figure 31. Path diagrams for models for the timing of the length of overwinter period of *C. newsteadi*. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the length of overwinter and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

5.3.4. *Culicoides pulicaris*

5.3.4.1. Seasonal Start PA

Accumulated degree days over 10ºC in winter showed a strong negative direct effect on the start of the season for both NF and PF (Fig. 32, A and B), therefore, sites with higher temperatures in winter had an earlier start of *C. pulicaris* season.

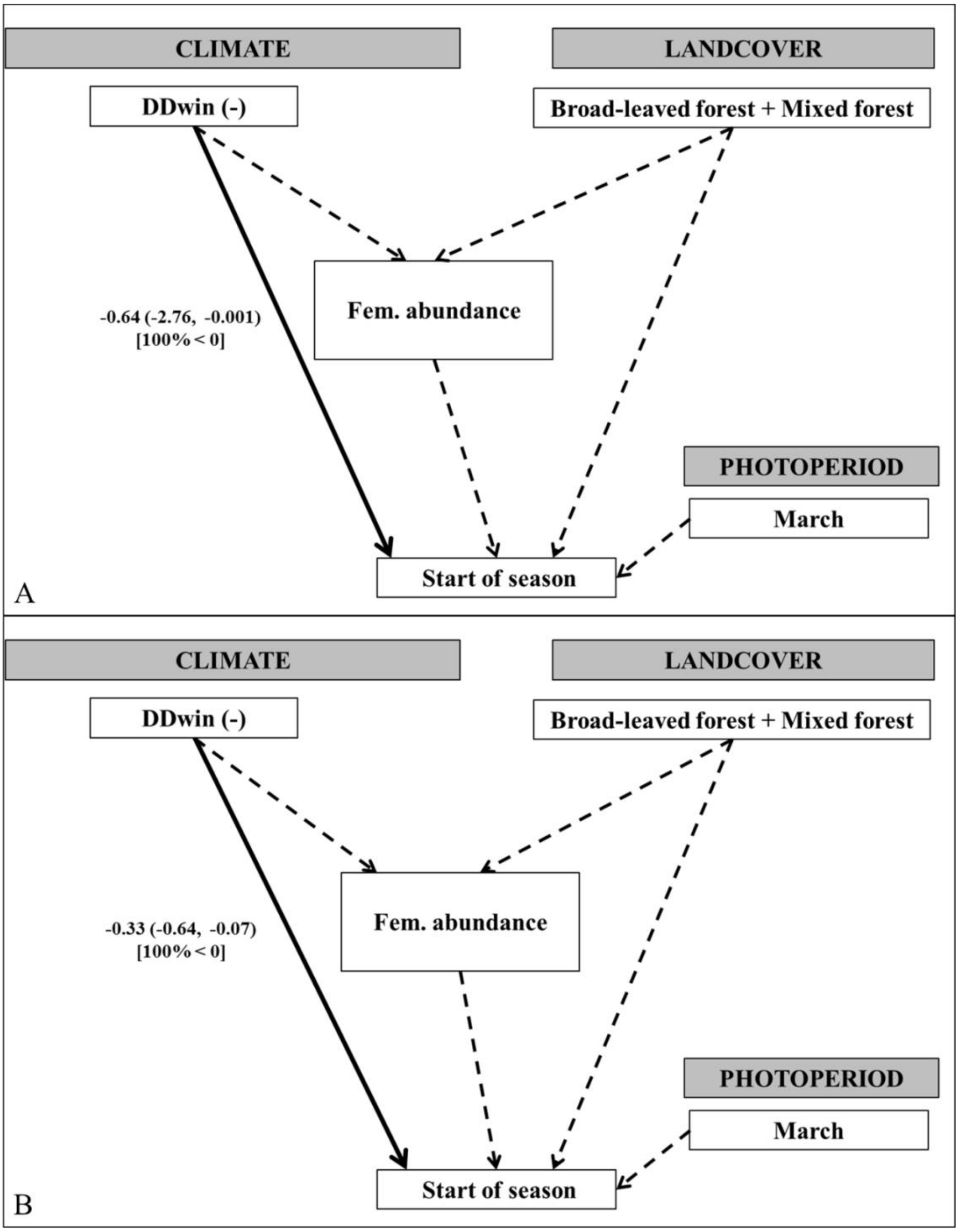


Figure 32. Path diagrams for models for the timing of the start of season of *C. pulicaris*. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while dotted lines represent no effect. Minus sign indicates a negative relationship between the timing of the start of season and the predictor variable. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are below zero.

5.3.4.2. Seasonal End PA

No environmental variables for the End of season model for both *C. pulicaris* NF and PF were included in the best model (Annex 15 and 24, null model).

5.3.4.3. Overwintering PA

Photoperiod in November in addition to the precipitation in autumn had a strong and weak positive effect respectively on the *C. pulicaris* length of overwinter period of NF (Fig. 33, A). In addition, the combination of pastures and natural grassland areas had a weak negative effect on NF abundance (Fig. 33, A). Therefore, sites with lower precipitation in autumn and a lower number of daylight hours during November were associated with shorter overwintering period and therefore a long period of activity of NF. Also, sites with smaller areas of pastures and natural grasslands were associated with a decrease in the number of NF as an indirect effect on the overwintering period of *C. pulicaris* NF. Regarding the PF, no variables of the overwintering period model showed strong or weak effects (Fig. 33, B).

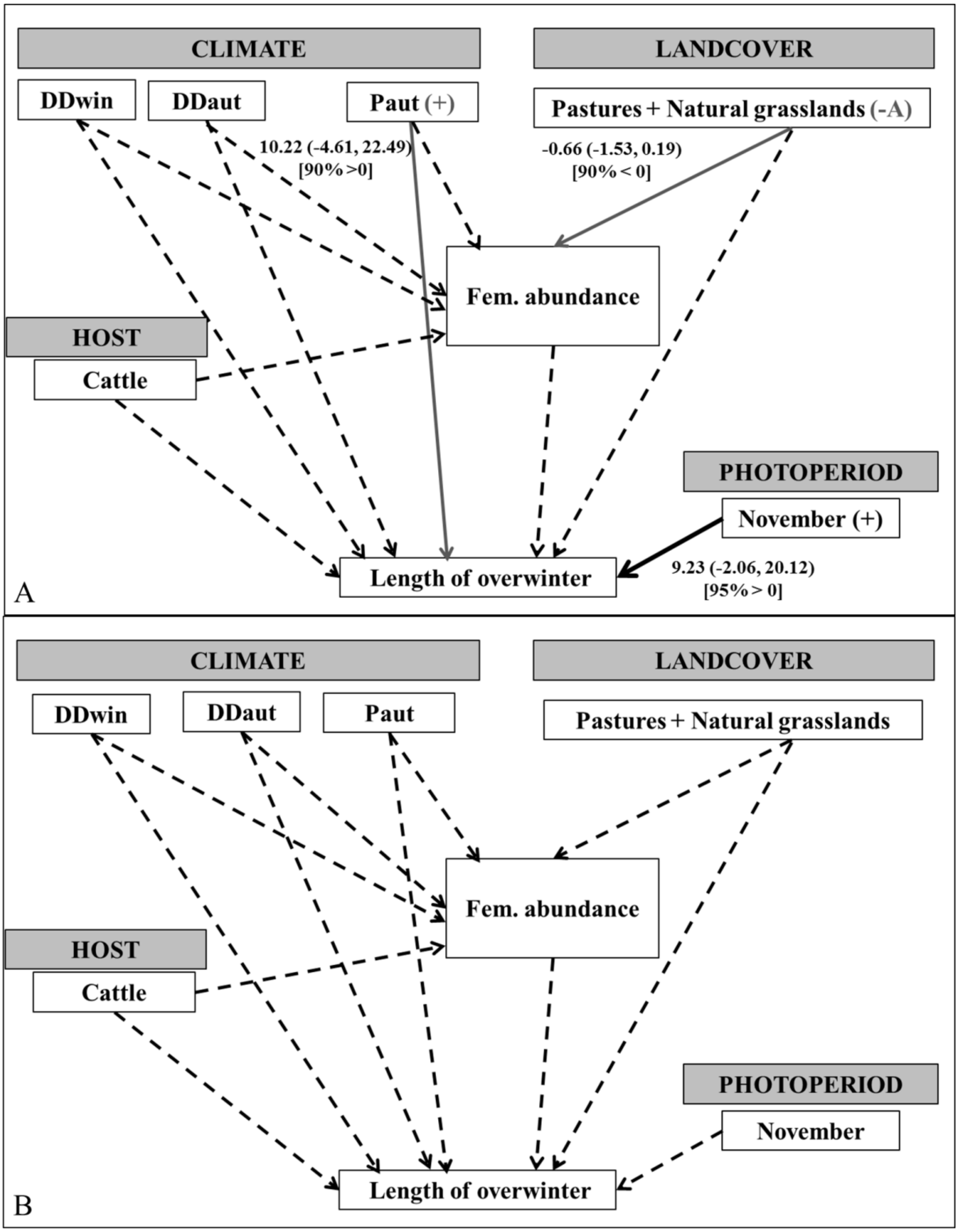


Figure 33. Path diagrams for models for the timing of the length of overwinter period of *C. pulicaris*. (A): nulliparous females (NF); (B): parous females (PF). Thick solid lines represent strong evidence for an effect (95 % credible interval does not overlap zero), while thin grey lines represent weak evidence for an effect (90 % credible interval does not overlap zero). Dotted lines represent no effect. Plus sign indicates a positive relationship between the timing of the length of overwinter and the predictor variable, while minus sign indicates negative relationship. Numbers on the unbroken arrows represent the posterior mean and credible interval and percentage of posterior values that are above or below zero.

The results of these models are summarized in tables 12 to 14 in order to have a global vision of the effects of variables on the seasonal activity of biting midges.

Culicoides imicola and Obsoletus complex were the species for which most significant environmental effects on seasonality were detected. The high *Culicoides* population was the variable with most significant effects among the species with the exception of *C. pulicaris*. This variable increased the activity period of NF and PF of all the taxa studied in the current study. The other environmental variables had different effects between life stages (NF or PF), especially for Obsoletus complex. In fact, the land cover, the topography and the photoperiod only had a significant effect on the PF of this species (Tables 12 and 13).

Similar to the previous chapter, the timing of the end of season only detected several significant environmental variables for two species, *C. imicola* and Obsoletus complex (Table 13). The reason was probably related to the higher variability across species of the timing of the end of the adult season versus the timing of the start of the season.

The environmental variables showed different effects on the overwinter periods between studied taxa (Table 11). *Culicoides imicola* overwintering was affected by temperature and precipitation during winter whereas Obsoletus complex was affected by the elevation and the photoperiod in November. For *C. newsteadi*, length of overwinter was only affected by the abundance of females. Meanwhile, *C. pulicaris* overwinter was only affected by the photoperiod in November.

Regarding the indirect effect via *Culicoides* females abundance, only the schlerophyllous vegetation had a strong significant effect on the start of the season of *C. imicola* NF via females abundance (Table 12). Conversely, precipitation in winter had a weak significant indirect effect on the start of the season of *C. newsteadi* NF via females abundance (Table 12), whereas Pastures and Natural grassland areas had a weak significant indirect effect on the overwinter period of *C. pulicaris* NF (Table 14).

Table 12. Summary of the significant environmental parameters for Start of the season models of each species. NF: Nulliparous females, PF: Parous females. Light grey: Variables included in the best model, dark grey: variable with weak effect (90% of credible interval did not include zero), black: Variable with strong effect (95% of credible interval did not include zero). (+): Positive effect, (-): Negative effect, (A): Indirect effect via *Culicoides* female abundance.

Species	**Stage**	**Elev**	**Cattle**	**Sheep**	**AgFor**	**NatGras**	**SchVeg**	**BrdMix**	**DDwin**	**DDspr**	**Pwin**	**Pspr**	**Phmar**	**Phapr**	**Fem. abund.**
C. imicola	NF				░		(+A)		░	░	░			░	░
	PF														
Obsoletus complex	NF		(-)			░				(-)		(-)	░		(-)
	PF	(+)	(+)							(-)		░		(-)	(-)
C. newsteadi	NF														
	PF			(+)					(-)		(-A)	░	░		(-)
C. pulicaris	NF							░	(-)				░		░
	PF							░	(-)				░		░

Table 13. Summary of the significant environmental parameters for End of the season models of each species. NF: Nulliparous females, PF: Parous females. Light grey: Variables included in the best model, dark grey: Variable with weak effect (90% of credible interval did not include zero), black: Variable with strong effect (95% of credible interval did not include zero). (+): Positive effect, (-): Negative effect, (A): Indirect effect via *Culicoides* female abundance.

Species	**Stage**	**Elev**	**Slope**	**Cattle**	**Sheep**	**NatGras**	**AgFor**	**SchVeg**	**BrdMix**	**DDaut**	**DDsum**	**Paut**	**Phsep**	**Phnov**	**Fem. abund.**
C. imicola	NF	(-)		(-)		░	░				(-)	░	░		░
	PF	(-)		░		(+)	░				(-)	░	░		░
Obsoletus complex	NF		░	(+)				░		░			░		(+)
	PF		(+)	(+)				(-)		(+)				░	(+)
C. newsteadi	NF														
	PF														
C. pulicaris	NF														
	PF														

Table 14. Summary of the significant environmental parameters for Length of overwinter of each species. NF: Nulliparous females, PF: Parous females. Light grey: Variables included in the best model, dark grey: Variable with weak effect (90% of credible interval did not include zero), black: Variable with strong effect (95% of credible interval did not include zero). (+): Positive effect, (-): Negative effect, (A): Indirect effect via *Culicoides* female abundance.

Species	**Stage**	**Elev**	**Slope**	**Cattle**	**NatGras**	**PastGras**	**AgFor**	**SchVeg**	**DDwin**	**DDaut**	**Pwin**	**(DDwin*Pwin)**	**Paut**	**Phsep**	**Phnov**	**Fem. abund**
C. imicola	NF				░		░		░	▒ (+)	░	█ (+)	░			░
	PF				░		░		░	░	▒ (-)	█ (+)	░			▒ (-)
Obsoletus complex	NF	█ (+)			░				░				░		█ (+)	░
	PF															
C. newsteadi	NF		▒ (+)	░			░	▒ (-)		░	░					█ (-)
	PF		░	░				░		░	░					▒ (-)
C. pulicaris	NF			░		▒ (-A)			░	░			▒ (+)		█ (+)	░
	PF			░		░			░	░			░	░	░	░

5.4. Discussion

This study has allowed us to quantify some of the environmental drivers involved in the phenology metrics for NF and PF of four different *Culicoides* vector species in Spain being of special interest for the determination of the SVFP. In addition, PA helped us to separate environmental effects on female abundance, narrowing down which environmental variables affect seasonality.

PA has shown for the first time the indirect effects of environmental variables through the *Culicoides* female abundance. However, only a few indirect effects were detected in our results where only the *C. imicola* NF start of season models showed strong indirect effects via female abundance. These unexpected results might be related to the accuracy of measures of the phenology and the *Culicoides* population size, which may not precisely reflect true values because of the inherent variability in weekly trap data. Future research with richer measures of phenology and population (e.g. daily rather than weekly traps) could improve the present results.

Results recorded from Acevedo *et al*. (2010) showed that the seasonality of *C. imicola* was determined by climate, topography, land cover and photoperiod, similar to the results obtained in the present study. The current study indicated that lower temperatures during summer prolonged the end of the species season, aligning with the findings that this species is more abundant in sites with lower altitudes and extensive plains recorded in that of Conte *et al*. (2003, 2007) and Torina (2004). The PA indicated the potential importance of land cover for the seasonality of this species: schlerophyllous vegetation had an indirect effect on the phenology through the increase of NF abundance. In fact, NF of that species seems to finish its season earlier when the cattle density increases, possibly because blood meals will be more widely available and NF will be more likely to develop into PF. Otherwise, the period of adult activity of *C. imicola* was negatively affected by the precipitation in winter and spring, probably due to the lower density of this species during those seasons and the dry preferences of the immature stages (Conte *et al*. 2007, Foxi and Delrio 2010).

Best models for PF of *C. imicola* start of season did not include any variable suggesting that other unmeasured environmental factors such as remotely sensed climatic variables (e.g. land surface temperature (LST) or enhanced vegetation index (EVI) (Purse *et al*. 2004a, 2004b, 2012) must be considered as drivers of the phenology and abundance of PF of this species.

Results obtained from the PA of Obsoletus complex species showed that temperature and precipitation play an important role on the seasonality of this species. The models showed that females may begin their season earlier when temperature increases in spring, coinciding with its peak of abundance in Spain (Ortega *et al*. 1998, 1999, Miranda *et al*. 2004) and consistent with the models recorded in that of White *et al.* (2017). Furthermore, higher precipitation in spring probably increase the humidity and breeding sites inducing the beginning of NF season due to the favorable conditions for its development. Different to *C. imicola*, schlerophyllous vegetation had a negative effect on the seasonality of Obsoletus complex species, making the end of the season earlier of this species, demonstrating that these two species do not share preferences for this habitats (Conte *et al*. 2007). We mentioned that photoperiod seemed to be an insignificant parameter in other studies with Obsoletus complex species. These studies were based on results from different treatments combining photoperiod and temperature for rearing *C. chiopterus* and *C. dewulfi* in laboratory conditions (Lühken *et al*. 2015) or other studies using the photoperiod as a predictor in statistical models for males and females of *C. obsoletus*, *C. scoticus*, *C. dewulfi* and *C. chiopterus* (Searle *et al*. 2014). However, our results showed that the number of daylight hours in April increased the active season of this species which agrees with other statistical studies like Searle *et al*. (2012) where the photoperiod was an important for other *Culicoides* species like *C. pulicaris*. Conversely, PA showed that the photoperiod in November increased the overwinter season of Obsoletus complex NF. This result was unexpected. A possible explanation remains unclear but could be related to the induction of overwintering since November is considered a month with lower captures of this species (Miranda *et al*. 2004, Conte *et al*. 2007, Talavera *et al*. 2015, Cuellar *et al*. 2018a, 2018b).

The elevation also played an important role for *C. imicola* and Obsoletus complex species and results from this chapter and the previous one (Tables 10-12) showed that season of Obsoletus complex species started earlier in lower elevation sites. However, PF of this species seems to be favoured by higher sloping sites increasing its adult activity period as recorded by other works like Capela *et al* (2003), Torina *et al*. (2004) and Conte *et al*. (2007). Obsoletus complex species are highly abundant in sites with wild ruminants and also livestock farms, suggesting the mammalophilic behaviour of this species complex (Talavera *et al*. 2015). Our results demonstrated that this species has long adult activity periods in sites with high cattle livestock in agreement with models from the previous chapter where the end of the season was extended in sites with high population of cattle. Conversely, PF started later in sites with high density of cattle livestock. This result was unexpected and further interactions with other variables such as cattle distribution in sites at a range of elevation should be considered to understand the factors affecting the Obsoletus complex distribution and seasonality. Best models for PF of Obsoletus complex species did not include any environmental driver for the end of the season suggesting that other variables such the aforementioned LST, EVI (Purse *et al*. 2012) or also other remotely sensed imagery data e.g. the normalized difference vegetation index (NDVI), the middle infra-red reflectance of the land cover (MIR) and the air temperature a few meters above ground (TAIR) (Purse *et al*. 2004a, 200b) must be considered in order to point out the role of the environment effects on the phenology and abundance of PF of this species.

Regarding *C. newsteadi*, results showed that this species was strongly affected by the abundance of females and, with weak effects, the temperatures, the topography, the land cover and livestock. The PA suggested that sites with lower slopes had shorter overwinter period, similar than results recorded by Torina *et al*. (2004) where *C. newsteadi* was more abundant in sites with low altitude. The temperature during winter was also significantly important for PF of this species which usually appear during seasons with high minimum temperatures, consistent with previous studies that have found a positive effect of high winter temperatures on abundance (Purse *et al*. 2004a, Ortega *et al*. 1999). In fact, PA results showed that high precipitation in winter had an indirect weak negative effect via females abundance on the start of the season of *C. newsteadi* NF, coinciding with the dry preferences of the south-western species (Ducheyne *et al*. 2013). The short overwinter period of *C. newsteadi* NF on schlerophyllous vegetation could be related to its breeding sites preferences, supporting the idea commented in the previous chapter about the suitability of schlerophyllous vegetation as habitat for breed (Foxi and Delrio 2010, González *et al*. 2013b). Livestock also played an important role. The season of *C. newsteadi* PF started later in sites with higher sheep densities. This result was unexpected since *C. newsteadi* prefers sheep as a host (Garros *et al*. 2011, Calvo *et al*. 2012, Martínez-de la Puente *et al*. 2015, Slama *et al*. 2015) and early stages of this species breed in sheep livestock holdings (Foxi and Delrio 2010, González *et al*. 2013b). In fact, this result was contradictory to the previous chapter where *C. newsteadi* total females started earlier in sites with higher density of sheep. A possible explanation was unclear but could be probably related to the distribution of the midges and the livestock across Spain. Best models for both NF of *C. newsteadi* start of season and NF and PF of *C. newsteadi* end of the season did not include any variables, suggesting that other environmental drivers must be considered in order to find how the environment affects the phenology and abundance of this species.

The PA models for *C. pulicaris* showed similar lack of significant environmental variables affecting the phenology of this species. Higher temperatures during winter was the only variable associated with an earlier start of the *C. pulicaris* season which could be related with the close relationship of that species with temperatures in cold months (Sanders *et al*. 2011, Kameke *et al*. 2017). In fact, L4 instar larvae of these insects cease diapause and continue development when temperature increases, thus synchronising the adult emergence in spring (White *et al*. 2017). Otherwise, PA has contributed to a better understanding of the overwinter period of *C. pulicaris*; the length of the activity period of NF seems to be favoured by high precipitation during autumn consistent with previous studies that found positive effects of this variable on the abundance and seasonality of *C. pulicaris* (Purse *et al*. 2004a, Searle *et al*. 2012, Ducheyne *et al*. 2013). Sites with pastures and natural grassland areas showed a weak negative effects on the length of overwinter of *C. pulicaris* NF via females abundance, possibly because these land covers may contain less suitable microhabitats for the early stages development of this species which usually breed in marshes or wetlands (Harrup *et al*. 2013, Zimmer *et al*. 2014b). The photoperiod in November plays also an important role in the phenology of this species (Searle *et al*. 2012) and our results showed that this variable increased the length of the overwinter period of NF.

Models for both NF and PF of *C. pulicaris* did not include any of the measured environmental variables for the end of the season. Patchy and uneven distribution of this species late in the season may preclude us from finding significant relationship with the variables we used in the models.

As well as PA of the current study has been a useful approach for understanding seasonality of insect vectors, PA performed in other aforementioned studies such as that of Fergnani *et al*. (2008) helped to determine that temperature was the only climatic variable that affected ant abundance directly whereas variation of the abundance was indirectly affected by the amount of resources. Conversely, the study of Mysterud *et al*. (2008) demonstrated that red deer were affected indirectly through trophic interactions and the results did not find evidences of direct effects on body mass. Further studies with other vectors such as mosquitoes could be interesting to improve the knowledge of their seasonality.

5.5. Conclusions

This study provides useful and valuable information and a PA framework for understanding how environmental drivers modulate the phenology and the abundance of *Culicoides* vector species in Spain. It has been demonstrated that the different *Culicoides* species respond to different environmental variables dependent upon their biological requirements and life stage (NF or PF).

Therefore, all the environmental elements should be included in the determination of the SVFP of these species. Suitable areas for these vector species have a higher risk of BTV or another pathogen transmitted of these *Culicoides* species in Spain.

New unmeasured variables should be integrated and modelling can be combined with other empirical lab and field studies (e.g. studies of *Culicoides* early stages, substrates for oviposition, adult attraction by traps) in order to provide a holistic understanding of seasonal regulation of insect vector populations.

5.6. Annexes

Annex 14. Best Start of the season models for NF and PF of each *Culicoides* species. pD: Effective number of parameters in each model. DIC: Deviance Information Criterion. N: Number of samples.

Models	**Model**	**N**	**Sites**	**Years**	**Variables**	**Start of Season**		**Fem. abundance**		**Total**
						pD	**DIC**	**pD**	**DIC**	**DIC**
C. imicola	NF	49	31	6	DDwin + DDspr + Pwin + (DDwin*Pwin) + SchVeg + Phapr + Fem. abundance	15.59	131.91	25.88	112.75	244.66
	PF	52	34	6	Null model with no covariates (a = -3.49 ± 0.26)	18.50	119.281	25.90	118.46	237.74
Obsoletus complex	NF	72	46	6	DDspr + Pspr + Cattle + NatGras + Phmarch + Fem. abundance	8.92	166.68	48.10	52.74	219.42
	PF	78	49	6	DDspr + Pspr + Elev + Cattle + Phapr + Fem. abundance	26.11	148.67	20.77	220.44	369.11
C. newsteadi	NF	45	29	5	Null model with no covariates (a = -3.70 ± 0.33)	6.79	118.05	27.34	63.74	181.79
	PF	51	34	5	DDwin + Pwin + Pspr + Sheep + Phmarch + Fem. abundance	27.85	111.69	31.20	88.94	200.63
C. pulicaris	NF	37	24	5	DDwin + BrdMix + Phmarch + Fem. abundance	4.91	115.60	13.69	101.38	216.97
	PF	37	24	5	DDwin + BrdMix + Phmarch + Fem. abundance	10.68	73.77	14.37	100.17	173.94

Annex 15. Best End of the season models for NF and PF of each *Culicoides* species. pD: Effective number of parameters in each model. DIC: Deviance information criterion. N: Number of samples.

Models	**Model**	**N**	**Sites**	**Years**	**Variables**	**End of Season**		**Fem. abundance**		**Total**
						pD	**DIC**	**pD**	**DIC**	**DIC**
C. imicola	NF	49	31	6	DDsum + Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	28.60	56.92	26.73	115.97	172.89
	PF	52	34	6	DDsum + Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	23.06	51.94	29.05	117.77	169.71
Obsoletus complex	NF	72	46	6	DDaut + Slope + Cattle + SchVeg + Phsep + Fem. abundance	19.31	209.83	46.76	61.53	271.36
	PF	78	49	6	DDaut + Slope + Cattle + SchVeg + Phnov + Fem. abundance	25.87	175.17	19.99	227.04	402.21
C. newsteadi	NF	45	29	5	Null model with no covariates (a = -2.72 ± 0.55)	5.49	110.64	27.54	63.81	174.46
	PF	51	34	5	Null model with no covariates (a = -1.95 ± 0.46)	10.61	135.71	30.16	90.91	226.63
C. pulicaris	NF	37	24	5	Null model with no covariates (a = -3.00 ± 0.49)	13.50	106.75	12.27	99.10	205.85
	PF	37	24	5	Null model with no covariates (a = -2.36 ± 0.51)	10.35	93.80	12.96	98.14	191.94

Annex 16. Best Length of Overwinter models for NF and PF of each *Culicoides* species. pD: Effective number of parameters in each model. DIC: Deviance information criterion. OW: Days overwintering. N: Number of samples.

Models	**Model**	**N**	**Sites**	**Years**	**Variables**	**Length of OW**		**Fem. abundance**		**Total DIC**
						pD	**DIC**	**pD**	**DIC**	
C. imicola	NF	27	17	5	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Fem. abundance	15.43	212.12	13.55	43.98	256.10
	PF	27	17	5	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Fem. abundance	16.48	181.70	14.24	45.11	226.81
Obsoletus complex	NF	32	25	5	DDwin + Paut + Elev + NatGras + Phnov + Fem. abundance	16.40	250.72	15.64	51.20	301.93
	PF	36	27	5	Null model with no covariates (a = -3.09 ± 0.57)	19.36	275.87	18.98	67.11	342.98
C. newsteadi	NF	27	19	4	DDaut + Slope + Pwin + Cattle + AgFor + SchVeg + Fem. abundance	13.98	227.78	16.93	15.13	242.92
	PF	27	19	4	DDaut + Slope + Pwin + Cattle + SchVeg + Fem. abundance	14.10	213.68	17.47	25.71	239.39
C. pulicaris	NF	21	16	4	DDwin + DDaut + Paut + Cattle + PastGras + Phnov + Fem. abundance	12.33	172.45	8.23	12.53	184.98
	PF	21	16	4	DDwin + DDaut + Paut + Cattle + PastGras + Phnov + Fem. abundance	11.51	162.50	8.12	28.02	190.52

Annex 17. Top 3 Start of the season models for *C. imicola* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 20 individuals per trap. N pred.: Number of predictors used in the model.

Models	N pred.	Variables	Total pD	Total DIC	Δ DIC
C. imicola **NF** (n= 49, 31 sites, 6 years)	7	DDwin + DDspr + Pwin + (DDwin*Pwin) + SchVeg + Phapr + Fem. abundance	41.47	244.66	-
	6	DDspr + Pwin + Cattle + SchVeg + Phapr + Fem. abundance	30.86	248.16	3.50
	6	DDspr + Pwin + AgFor + SchVeg + Phapr + Fem. abundance	35.70	250.25	5.59
	0	Null model with no covariates (a = -3.52 ± 0.27)	33.36	250.90	6.24
C. imicola **PF** (n= 52, 34 sites, 6 years)	0	Null model with no covariates (a = -3.49 ± 0.26)	44.4	237.74	-
	6	DDspr + Pwin + AgFor + SchVeg + Phapr + Fem. abundance	52.25	239.61	1.87
	6	DDspr + Pwin + AgFor + SchVeg + Phapr + Fem. abundance	50.68	243.15	3.54
	7	DDwin + DDspr + Pwin + (DDwin*Pwin) + SchVeg + Phapr + Fem. abundance	45.47	250.70	11.09

Annex 18. Top 3 Start of the season models for Obsoletus complex NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 1 individuals per trap. N pred.: Number of predictors used in the model.

Models	N pred.	Variables	Total pD	Total DIC	Δ DIC
Obsoletus complex NF (n= 72, 46 sites, 6 years)	6	DDspr + Pspr + Cattle+ NatGras + Phmarch + Fem. abundance	57.02	219.42	-
	6	DDspr + Pspr + Cattle + SchVeg + Phmarch + Fem. abundance	61.70	223.04	3.62
	6	DDspr + Pspr + Cattle + SchVeg + Phapr + Fem. abundance	62.54	227.86	8.44
	0	Null model with no covariates (a = -4.00 ± 0.28)	70.88	251.81	32.39
Obsoletus complex PF (n= 78, 49 sites, 6 years)	6	DDspr + Pspr + Elev + Cattle + Phapr + Fem. abundance	46.88	369.11	-
	6	DDspr + Pspr + Elev + Cattle + Phmarch + Fem. abundance	49.50	374.51	5.40
	6	DDspr + Pspr + Cattle + SchVeg + Phapr + Fem. abundance	55.30	377.75	8.64
	0	Null model with no covariates (a = -4.17 ± 0.23)	49.85	380.28	11.17

Annex 19. Top 3 Start of the season models for *C. newsteadi* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
C. newsteadi **NF** (n= 45, 29 sites, 5 years)	0	Null model with no covariates (a = -3.70 ± 0.33)	34.13	181.79	-
	6	DDwin + Pwin + Pspr + Sheep + Phmarch + Fem. abundance	44.78	186.90	5.11
	6	DDwin + Pwin + Pspr + Elev + Slope + Fem. abundance	47.56	196.90	15.11
	6	DDwin + DDspr + Pspr + Elev + AgFor + Fem. abundance	45.00	200.28	18.49
C. newsteadi **PF** (n= 51, 34 sites, 5 years)	6	DDwin + Pwin + Pspr + Sheep + Phmarch + Fem. abundance	59.05	200.63	-
	7	DDwin + Elev + Slope + Pwin + Pspr + Sheep + Fem. abundance	58.60	202.28	1.21
	8	DDwin + Elev + Slope + Pwin + Pspr + Cattle + Fem. abundance	59.19	202.87	1.80
	0	Null model with no covariates (a = -3.69 ± 0.22)	55.32	208.12	7.49

Annex 20. Top 3 Start of the season models for *C. pulicaris* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. ∆ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**∆ DIC**
C. pulicaris **NF** (n= 37, 24 sites, 5 years)	4	DDwin + BrdMix + Phmarch + Fem. abundance	18.60	216.97	-
	0	Null model with no covariates (a = -3.74 ± 0.33)	18.56	218.40	1.43
	3	DDwin + Phmarch + Fem. abundance	21.48	220.25	3.28
	5	DDwin + (DDwin*Pwin) + BrdMix + PastGras + Phmarch + Fem abundance	19.48	220.56	3.59
C. pulicaris **PF** (n= 37, 24 sites, 5 years)	4	DDwin + BrdMix + Phmarch + Fem. abundance	25.05	173.94	-
	4	DDwin + Sheep + Phmarch + Fem. abundance	25.06	174.95	1.01
	4	DDwin + Cattle + Phmarch + Fem. abundance	24.64	175.03	1.09
	0	Null model with no covariates (a = -4.10 ± 0.28)	22.15	188.58	14.64

Annex 21. Top 3 End of the season models for *C. imicola* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
C. imicola **NF** (n= 49, 31 sites, 6 years)	8	DDsum + Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	55.33	172.89	-
	9	DDaut + DDsum + Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	56.22	176.98	4.09
	7	DDaut + Elev + Paut + Cattle + NatGras + Phsep + Fem. abundance	54.63	179.25	6.36
	0	Null model with no covariates (a = -1.39 ± 0.41)	44.29	190.58	17.69
C. imicola **PF** (n= 52, 34 sites, 6 years)	8	DDsum + Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	51.84	169.11	-
	7	Elev + Paut + Cattle + AgFor + NatGras + Phsep + Fem. abundance	52.11	169.71	0.60
	7	DDaut + Elev + Paut + Cattle + NatGras + Phsep + Fem. abundance	51.19	171.72	2.61
	0	Null model with no covariates (a = -1.08 ± 0.33)	38.44	182.98	13.87

Annex 22. Top 3 End of the season models for Obsoletus complex NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	N pred.	Variables	Total pD	Total DIC	Δ DIC
Obsoletus complex NF (n= 72, 46 sites, 6 years)	6	DDaut + Slope + Cattle + SchVeg + Phsep + Fem. abundance	66.07	271.36	-
	5	DDaut + Slope + Cattle + Phsep + Fem. abundance	66.05	272.08	0.72
	0	Null model with no covariates (a = -2.03 ± 0.50)	62.96	272.34	0.98
	5	DDaut + Slope + Cattle + Phnov + Fem. abundance	65.53	272.43	1.07
Obsoletus complex PF (n= 78, 49 sites, 6 years)	6	DDaut + Slope + Cattle + SchVeg + Phnov + Fem. abundance	44.86	402.21	-
	6	DDaut + Slope + Cattle + SchVeg + Phsep + Fem. abundance	44.48	402.92	0.71
	6	DDaut + Slope + Cattle + NatGras + Phnov + Fem. abundance	45.97	407.08	4.87
	0	Null model with no covariates (a = -1.66 ± 0.30)	42.80	413.77	11.56

Annex 23. Top 3 End of the season models for *C. newsteadi* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
C. newsteadi **NF** (n= 45, 29 sites, 5 years)	0	Null model with no covariates (a = -2.72 ± 0.55)	33.03	174.46	-
	5	DDaut + Psum + (DDaut*Psum) + Phsep + Fem. abundance	50.46	183.01	8.55
	6	DDaut + DDsum + Psum + (DDaut*Psum) + Phnov + Fem. abundance	50.14	186.13	11.67
	6	DDaut + DDsum + Psum + (DDaut*Psum) + Phsep + Fem. abundance	48.32	189.73	15.27
C. newsteadi **PF** (n= 51, 34 sites, 5 years)	0	Null model with no covariates (a = -1.95 ± 0.46)	40.77	226.63	-
	5	DDaut + Psum + (DDaut*Psum) + Phnov + Fem. abundance	45.65	228.07	1.44
	5	DDsum + Psum + (DDaut*Psum) + Phsep + Fem. abundance	45.29	229.68	3.05
	5	DDaut + Psum + (DDaut*Psum) + Phsep + Fem. abundance	44.98	230.21	3.58

Annex 24. Top 3 End of the season models for *C. pulicaris* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. ∆ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**∆ DIC**
C. pulicaris **NF** (n= 37, 24 sites, 5 years)	0	Null model with no covariates (a = -3.00 ± 0.49)	25.77	205.85	-
	6	DDsum + Psum + (DDsum*Psum) + Sheep + Phsep + Fem. abundance	28.97	210.33	4.46
	7	DDsum + Psum + (DDsum*Psum) + Sheep + AgFor Phsep + Fem. abundance	29.83	211.71	5.86
	6	DDsum + Psum + (DDsum*Psum) + Sheep + Phnov + Fem. abundance	31.99	218.26	12.41
C. pulicaris **PF** (n= 37, 24 sites, 5 years)	0	Null model with no covariates (a = -2.36 ± 0.51)	23.31	191.94	-
	6	DDsum + Psum + (DDsum*Psum) + Sheep + Phsep + Fem. abundance	31.83	199.29	7.35
	6	DDsum + Psum + (DDsum*Psum) + Cattle + Phsep + Fem. abundance	32.61	199.35	7.41
	7	DDsum + Psum + (DDsum*Psum) + Sheep + PastGras + Phsep + Fem. abundance	32.26	199.43	7.55

Annex 25. Top 3 Length of Overwinter models for *C. imicola* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
C. imicola **NF** (n= 27, 17 sites, 5 years)	8	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Fem. abundance	28.98	256.10	-
	6	DDwin + DDaut + Paut + AgFor + NatGras + Fem. abundance	30.35	267.80	11.70
	0	Null model with no covariates (a = -3.09 ± 0.57)	24.62	276.05	19.95
	6	DDwin + Pwin + Paut + AgFor + NatGras + Fem. abundance	28.99	278.09	21.99
C. imicola **PF** (n= 27, 17 sites, 5 years)	8	DDwin + DDaut + Pwin + Paut + (DDwin*Pwin) + AgFor + NatGras + Fem. abundance	30.72	226.81	-
	6	DDwin + DDaut + Pwin + AgFor + NatGras + Fem. abundance	29.49	247.69	20.88
	0	Null model with no covariates (a = -3.08 ± 0.57)	26.13	254.07	27.26
	6	DDwin + Pwin + Paut + AgFor + NatGras + Fem. abundance	30.42	255.98	29.17

Annex 26. Top 3 Length of Overwinter models for Obsoletus complex NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
Obsoletus complex NF (n= 32, 25 sites, 5 years)	6	DDwin + Paut + Elev + NatGras + Phnov + Fem. abundance	32.04	301.93	-
	6	DDaut + Paut + Elev + NatGras + Phnov + Fem. abundance	32.13	316.98	15.05
	0	Null model with no covariates (a = -3.09 ± 0.56)	31.72	318.42	16.49
	5	DDaut + Elev + NatGras + Phnov + Fem. abundance	31.16	319.01	17.08
Obsoletus complex PF (n= 26, 27 sites, 5 years)	0	Null model with no covariates (a = -3.09 ± 0.57)	38.34	342.98	-
	7	DDwin + Pwin + Elev + Cattle + NatGras + Phsep + Fem. abundance	38.82	343.88	0.90
	7	DDaut + Paut + Elev + Cattle + NatGras + Phnov + Fem. abundance	39.04	355.38	12.40
	7	DDaut + Paut + Elev + Cattle + NatGras + Phsep + Fem. abundance	39.24	357.58	14.60

Annex 27. Top 3 Length of Overwinter models for *C. newsteadi* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	**N pred.**	**Variables**	**Total pD**	**Total DIC**	**Δ DIC**
C. newsteadi **NF** (n= 27, 19 sites, 4 years)	7	DDaut + Pwin + Slope + Cattle + AgFor + SchVeg + Fem. abundance	30.91	242.92	-
	6	DDaut + Pwin + Slope + Cattle + AgFor + Fem. abundance	30.15	243.51	0.59
	7	DDaut + Pwin + Paut + Cattle + AgFor + SchVeg + Fem. abundance	30.10	249.91	6.99
	0	Null model with no covariates (a = -3.10 ± 0.57)	25.10	250.85	7.93
C. newsteadi **PF** (n= 27, 19 sites, 4 years)	6	DDaut + Slope + Pwin + Cattle + SchVeg + Fem. abundance	31.57	239.39	-
	7	DDaut + Slope + Pwin + Cattle + AgFor + SchVeg + Fem. abundance	32.67	240.20	0.81
	6	DDaut + Slope + Pwin + Cattle + AgFor + Fem. abundance	31.67	241.80	2.41
	0	Null model with no covariates (a = -3.10 ± 0.56)	24.05	244.28	4.89

Annex 28. Top 3 Length of Overwinter models for *C. pulicaris* NF and PF (best model in grey). a: Intercept. pD: Effective number of parameters in each model. DIC: Deviance information criterion. Δ DIC: Difference with the best model. n= Number of samples with > 5 individuals per trap. N pred.: Number of predictors used in the model.

Models	N pred.	Variables	Total pD	Total DIC	Δ DIC
C. pulicaris **NF** (n= 21, 16 sites, 4 years)	7	DDwin + DDaut + Paut + Cattle + PastGras + Phnov + Fem. abundance	20.56	184.98	-
	0	Null model with no covariates (a = -3.10 ± 0.56)	17.46	186.91	1.93
	6	DDaut + Paut + Cattle + AgFor + Phnov + Fem. abundance	18.79	189.72	4.74
	6	DDwin + DDaut + Paut + Cattle + AgFor + Fem. abundance	20.25	210.90	25.92
C. pulicaris **PF** (n= 21, 16 sites, 4 years)	7	DDwin + DDaut + Paut + Cattle + PastGras + Phnov + Fem. abundance	19.63	190.52	-
	7	DDwin + DDaut + Paut + Cattle + PastGras + Phnov + Fem. abundance	19.87	192.24	1.72
	0	Null model with no covariates (a = -3.10 ± 0.56)	16.41	201.79	11.27
	7	DDwin + DDaut + Paut + Cattle + AgFor + Phnov + Fem. abundance	21.12	205.48	14.96

6. Bionomics of livestock-associated *Culicoides* (biting midge) bluetongue virus vectors under laboratory conditions




































6. Bionomics of livestock-associated *Culicoides* (biting midge) bluetongue virus vectors under laboratory conditions

6.1. Introduction

Certain *Culicoides* species feed predominantly on domestic livestock (cattle, horses or sheep) and can become abundant on farms (Tabachnick 2004) and play a role in transmission of arboviral livestock diseases. The epidemiology of vector borne diseases is not only influenced by the climate but also by factors such as the life-cycle of the vector, vector establishment and abundance (Lysyk and Dank 2007, Verhoef *et al*. 2014). The persistence and abundance of domestic-host associated *Culicoides* species are governed by habitats around farm premises used as larval development sites by BTV vector species such as *C. imicola* and *C. obsoletus* (Mellor and Pitzolis 1979, Harrup *et al*. 2013, Purse *et al*. 2015).

To date, relative few *Culicoides* species e.g. *C. nubeculosus* and *C. sonorensis* have been successfully cultured in the laboratory (reviewed in Hunt 1994 and Carpenter 2001). Consequently, the bionomic characteristics of most of the important vector species have not been systematically quantified.

The most extensive bionomic studies on *Culicoides* were conducted by Becker (1960) on the feeding, mating and egg and larvae description of the non-vector species *C. circumscriptus* in Glasgow. Kettle (1962) published a review about mating, feeding and oviposition habits of the family Ceratopogonidae including the genera *Culicoides*, *Leptoconops* and *Forcipomyia*, and Nevill (1967) studied the basic bionomics of a group of species including *C. imicola* in South Africa. The study of Purse *et al*. (2015) reviewed the worldwide available information on *Culicoides* bionomics.

Some studies have focused on particular aspects of *Culicoides* bionomics. For instance, laboratory observations on the autogeny and horizontal migration and diet of *Culicoide*s *furens* Poey larvae in Florida and Jamaica (Linley 1966, Koch and Axtell 1978, Aussel and Linley 1994). The effect of temperature on the oogenesis and fecundity of *C. brevitarsis* (Campbell and Kettle 1975) and *C. impunctatus* (Carpenter *et al*. 2006b) were studied in Australia and Scotland respectively. The reproductive potential, seasonal fecundity and parity of *Culicoides melleus* (Coquillet) and *Culicoides insignis* Lutz were studied in Florida (Linley and Hinds 1976, Kramer *et al*. 1985) and that of *Culicoides occidentalis* Wirth & Jones in California (Smith and Mullens 2003). The adult feeding behaviour of *C. nubeculosus* and species from Pulicaris and Obsoletus groups has been studied in Switzerland (Jamnback 1961, Kaufmann *et al*. 2015) and Italy (Goffredo *et al*. 2004). The effects of temperature, photoperiod, flooding on breeding substrate and emergence of Obsoletus group larvae have been determined in Germany (Lühken *et al*. 2014a, 2014b, 2015, Steinke *et al*. 2015). Several studies determined the effects of temperature and humidity on the sub-adult stages, adult size, EIP, vector competence, and seasonality on *C. sonorensis* in UK (Colony), Denver, Canada and California (Akey *et al*. 1978, Mullens 1987, Lysyk and Danyk 2007, Wittmann *et al*. 2002, McDermott *et al*. 2016); *Culicoides arakawae* (Arakawa) and *Culicoides maculatus* Shiraki in Japan (Kitaoka 1982); and *C. imicola* and *C. bolitinos* in South Africa (Verhoef *et al*. 2014). Oviposition studies have been carried out in Israel on *C. imicola* (Braverman and Linley 1994) and in the Netherlands on *C. impunctatus* (Carpenter *et al*. 2001). The size of larvae (Kettle and Lawson 1952) and the vertical distribution of several *Culicoide*s sub-adult stages were determined in Turkey (Uslu and Dik 2006) and Germany (Lühken *et al*. 2014a).

Currently only two studies have explored the duration of sub-adult stages, oviposition and lifespan of *Culicoides* vectors: studies on *C. peregrinus* in India comparing various food sources and substrates (Harsha and Mazumdar 2015) and the contributions of Veronesi *et al*. (2009) on *C. imicola* in South Africa.

Artificial blood feeding is often used for *Culicoides* rearing under laboratory conditions. This technique allows to perform oral susceptibility and vector competence studies on vector species (Venter *et al*. 1991, 1998, 2003, Carpenter *et al*. 2006a, Del Río *et al*. 2012) as well as the maintenance of laboratory colonies (Hunt 1994, Carpenter *et al*. 2001, Goffredo *et al*. 2004, Veronesi *et al*. 2009).

The objective of the present study is to contribute to the knowledge on the bionomics under laboratory conditions of *C. imicola* and *C. obsoletu*s, considered the major European vectors of BTV. Other species included were *C. newsteadi*, recently incriminated as a potential vector of BTV (Goffredo *et al*. 2015, Foxi *et al*. 2016), as well as other ornithophilic non-vector species such as *Culicoides cataneii* Clastrier, *C. paolae* and *C. circumscriptus*. Experiments were also conducted by artificial feeding of *C. obsoletus* nulliparous adult females in order to compare it with the results obtained from field gravid females.

The study of bionomics in the laboratory will contribute to our understanding of parameters such as vectorial capacity, the basic reproduction ratio (R_0) and also the requirements for the establishment of a *Culicoides* spp. colony in laboratory conditions.

6.2. Material and methods

6.2.1. Samplings

Adult *Culicoides* were collected alive at a cattle farm on Majorca Island (‘Son Valls’, Felanitx, Balearic Islands, Spain; 39° 31' 21.12"N; 3° 6' 21.55"E) for 22 non-consecutive nights between April 8th and November 25th 2014 (No BTV was circulating during that period) with UV suction-light traps (Onderstepoort model; OVI-ARC, Onderstepoort, South Africa). Two traps, separated 30 metres from each other to avoid interference, were operated from dusk to dawn outside the stables. In order to increase the survival of the trapped midges, damp paper, providing humid shelter for *Culicoides* adults, was placed inside the collection pots.

6.2.2. Laboratory procedures

Once in the laboratory, catches were immediately emptied into a gauze cage. Active *Culicoides* midges were aspirated and immobilized in a freezer for one minute following the procedures of Venter *et al*. (1991). Afterwards, insects were transferred to a refrigerated chill table for selecting Obsoletus complex NF and *Culicoides* spp. gravid females. Selected females were kept individually in cylindrical 64 x 50 mm cardboard boxes (Watkins & Doncaster®, Herefordshire, UK) provided with a 5 cm diameter plastic Petri dish with filter paper and moistened cotton wool as substrate for oviposition at the bottom (Fig. 34). Females were fed using paper pads moistened with a mixture of 10% sucrose solution and 0.2% of Nipagin (Methyl *p*-hydroxybenzoate) as an antibiotic to prevent fungal growth (Veronesi *et al*. 2009).

Figure 34. Cardboard box with 5 cm plastic Petri dish at the bottom provided with moistened cotton wool and filter paper as substrate for oviposition of *Culicoides* females.

6.2.3. Artificial blood feeding procedures for Obsoletus complex NF

In order to prepare Obsoletus complex individuals for blood feeding, NF were kept in the same cardboard boxes provided with 10% sucrose solution and 0.2% of Nipagin (Methyl *p*-hydroxybenzoate) during 72 h followed by 24-hour starving period.

For the artificial blood feeding, fresh bovine blood obtained from the local slaughterhouse was defibrinated using 6 mm glass beads (Labbox®) in 250 ml containers (Fig. 35, A and B). The blood was drawn into the arms of a Hemotek© 5W1 (Discovery Workshops, Accrington Lancashire, UK) using Parafilm® “M” (Pechiney®, Chicago) as artificial membrane (Fig 36, A). The arm of the Hemotek© was then placed on top of the cardboard boxes where the Obsoletus complex individuals were lodged to feed for 45 minutes (Fig 36, B).

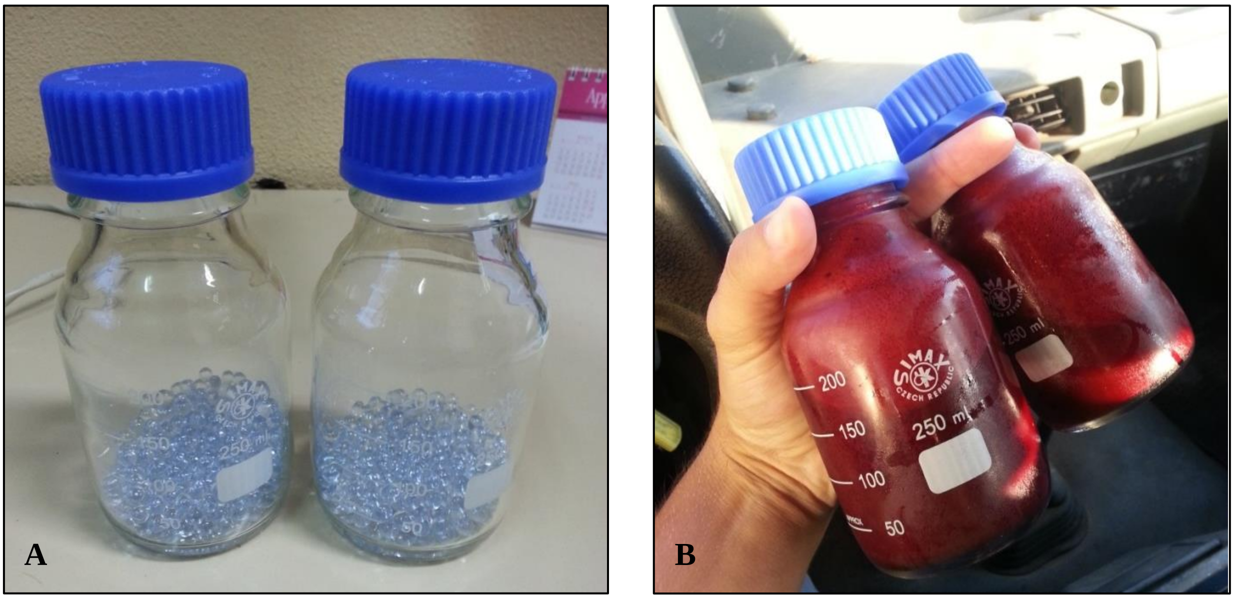


Figure 35. Glass beads (Labbox®) in 250 ml containers used for defibrinating blood (A). Containers filled with the glass beads and bovine blood (B).

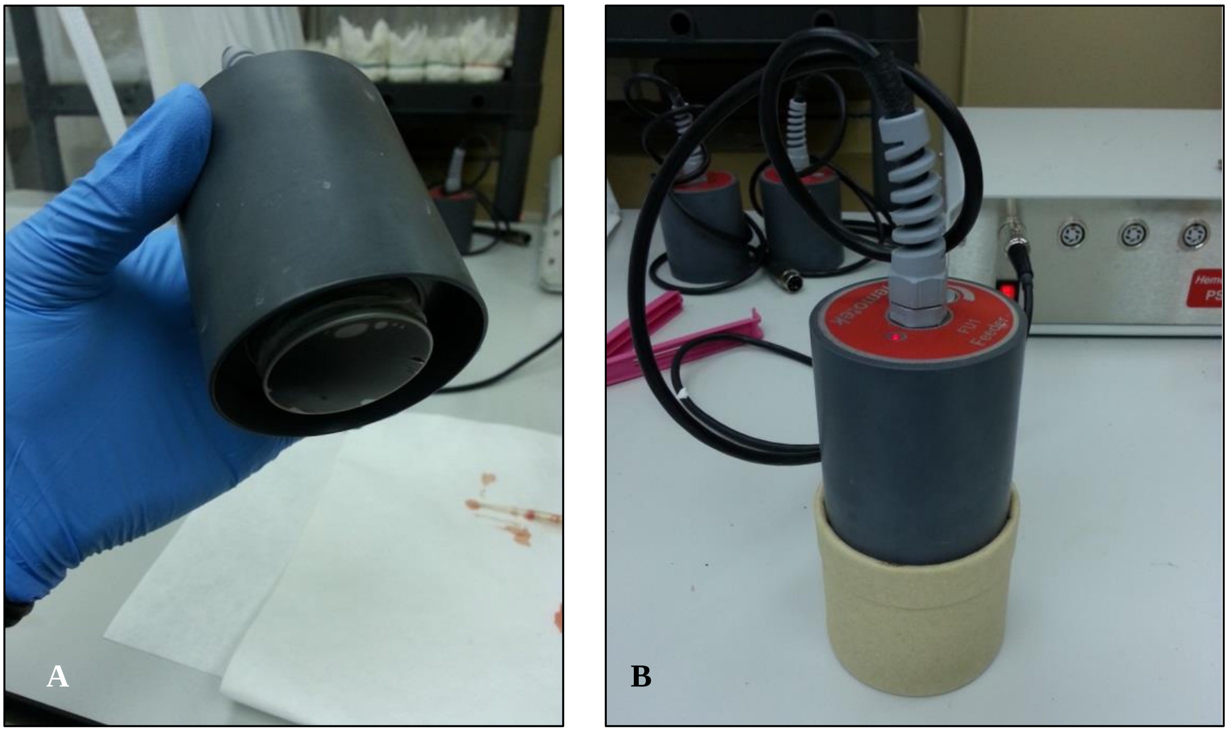


Figure 36. Parafilm® artificial membrane attached to the Hemotek© arm (A). Hemotek© arm set on top of the cardboard box with the *C. obsoletus* NF inside (B).

6.2.4. *Culicoides* rearing

After oviposition (Fig. 37), eggs were counted and transferred for hatching (Fig. 39) to 100 mm Petri dishes with 10 ml of 2% European Bacteriological Agar gel medium (CONDA® laboratories; Pronadisa, Spain) and humidified daily to prevent desiccation (Boorman 1985) (Fig. 38). The cardboard boxes and the Petri dishes were kept at 25 ± 1°C and 90 ± 2 % relative humidity (RH) in a climatic chamber (Climas® Grow chamber; Barcelona, Spain). Samples were initially kept at 12:12 h photoperiod, however, the increase of temperature inside the climatic chamber due to light tubes, forced us to keep the samples in the dark. Percentage of hatch per batch of eggs per female was recorded. Eggs that did not hatch within 3 weeks were discarded.

Figure 37. Example of oviposited eggs on the 5 cm plastic Petri dish at the bottom of cardboard boxes provided with moistened cotton wool and filter paper.

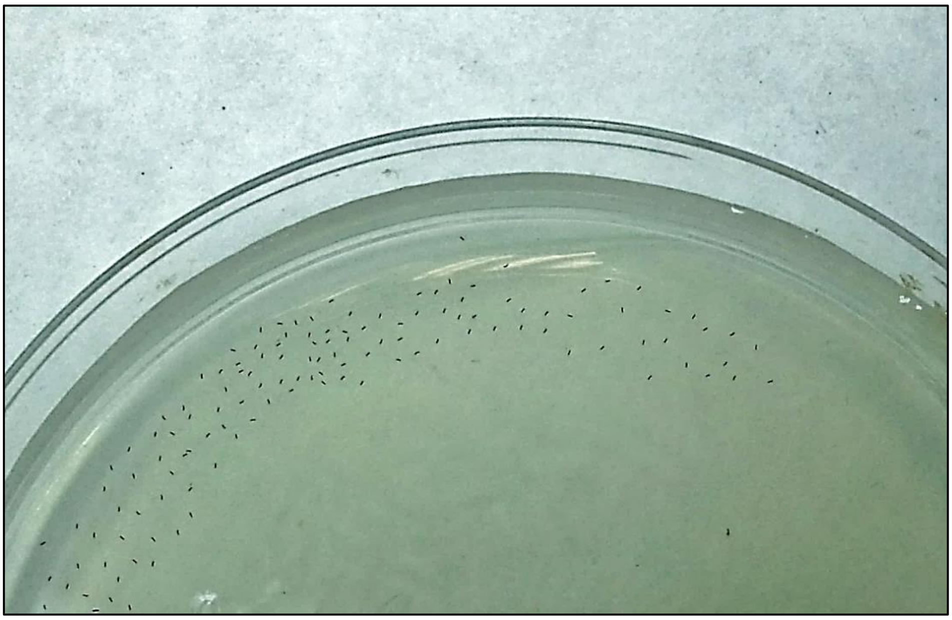


Figure 38. Oviposited eggs transferred to a to 100 mm Petri dishes with 10 ml of 2% European Bacteriological Agar gel medium.

Figure 39. First instar larvae just emerged from the egg on the agar gel medium. Hatched eggs and its opercles are also observed.

A Stereomicroscope (Nikon® SMZ 645) was used to daily observe the larvae and eggs in the Petri dishes. *Panagrellus redivivus* (L.) (Nematoda: Panagrolaimidae) grown in cereal powder was daily used to feed the *Culicoides* larvae (Fig. 40) (Kettle *et al*. 1975, Boorman 1985). Larval stages were recorded from each Petri dish according to the rank of the total length of larvae (Kettle and Lawson 1952, Becker 1960, Murphree and Mullen 1991). Pupae were counted and transferred again to the 5 cm Petri dishes with moistened filter paper in the cardboard boxes (max. 10 pupae / box) (Fig. 41, A and B) till adults emerged. Percentage of adult emerged from each *Culicoides* female progeny was recorded. The emerged adults were maintained in the cardboard boxes (Fig. 42, A and B) and fed daily with a 10% sugar solution to determine its lifespan.

Figure 40. Fourth instar *Culicoides* larvae feeding upon nematode (pointed with a white arrow) in agar gel medium.

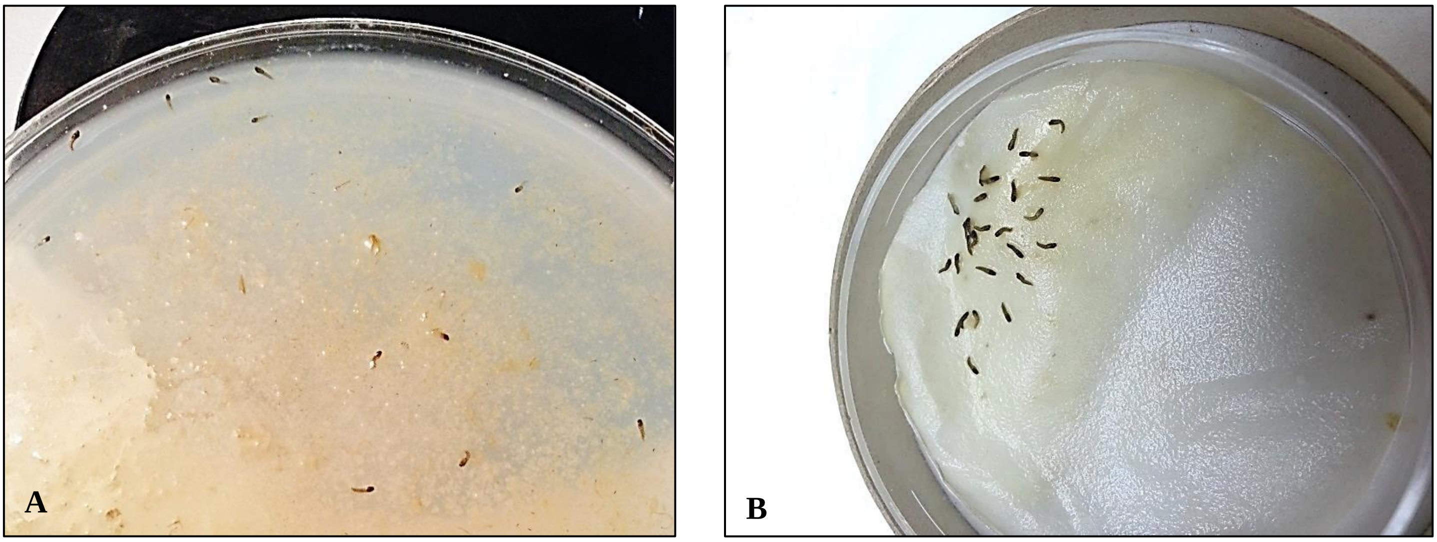


Figure 41. Pupae on the agar gel medium of the Petri dish (A). Pupae transferred to the Petri dish of the cardboard boxes for adult emergence (B).

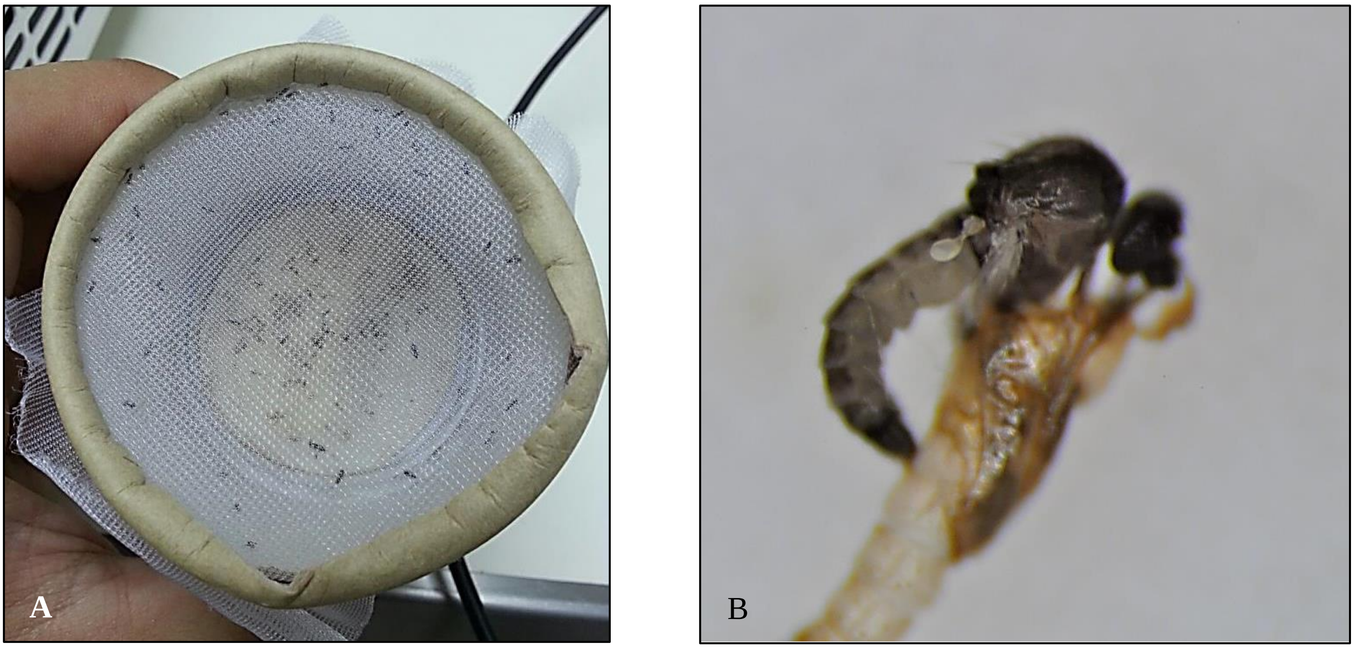


Figure 42. Emerged *Culicoides* individuals observed through the cardboard box mesh (A). *Culicoides* individual emerging from pupa (B).

Culicoides species were identified according to their wing pattern (Rawlings 1996, Mathieu *et al*. 2012). Due to the difficulty in identifying females of the Obsoletus complex species, we used the males on emergence to identify the species of this group following the keys of Campbell and Pelham-Clinton (1960), Delécolle (1985) and Nielsen and Kristensen (2011).

The SPSS Statistics® 17.0 software was used to analyse the data. Analyses were subjected to non-parametric Kolmogórov-Smirnov two-sample test (K-S) for assessing the differences of bionomic parameters between the Obsoletus complex artificial engorged females and the gravid females obtained from field.

6.3. Results

6.3.1. Species composition

A total of 1169 individuals were collected in traps, comprising 34.2% NF followed by a 32.6% of gravid females (Fig. 43). The predominant species were Obsoletus complex and *C. circumscriptus* representing the 63.2% and 21.3% of the whole sample respectively (Fig. 44).

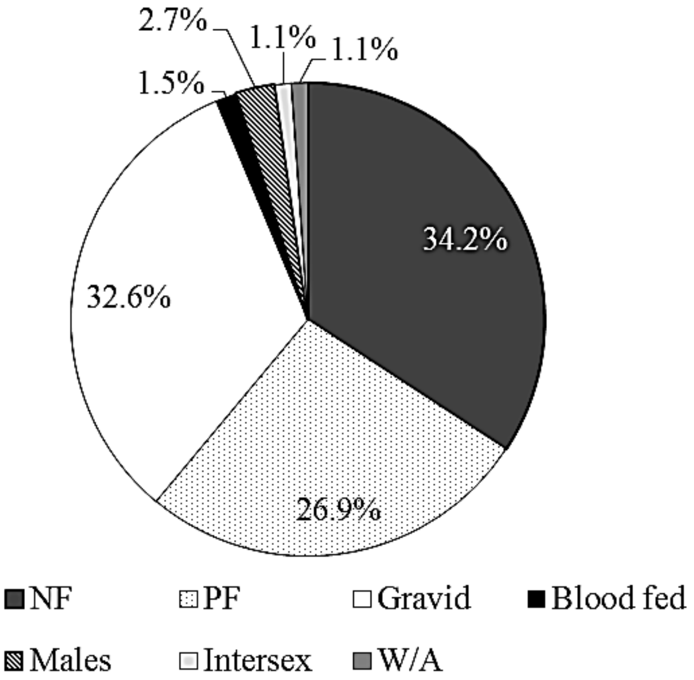


Figure 43. Percentages of different gonotrophic stages among the total *Culicoides* individuals collected from field. W/A (Without Abdomen): Individuals that had lost their abdomens such that gonotrophic stage was impossible to determine.

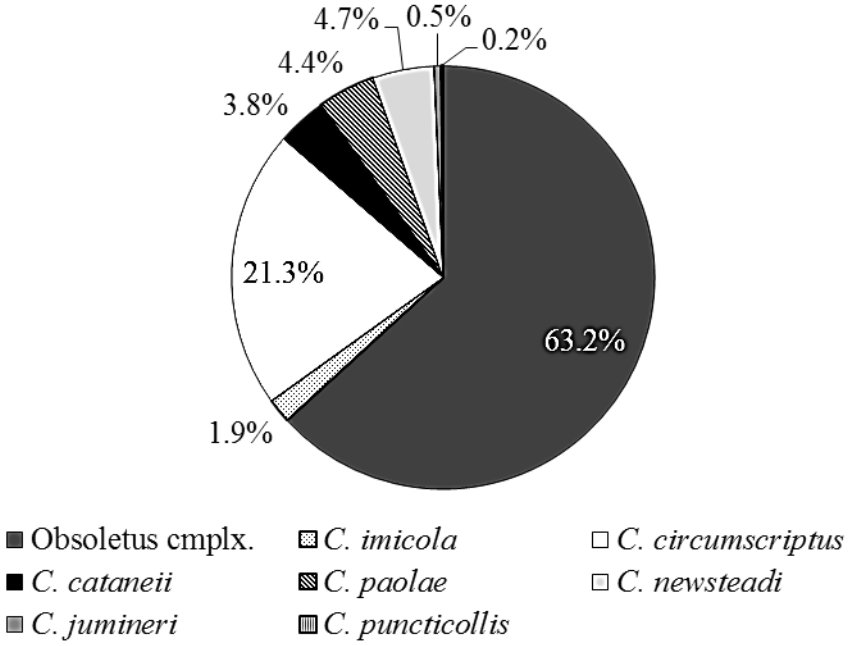


Figure 44. Percentage of species composition from the total *Culicoides* individuals (females and males) collected from field.

From this, 263 *Culicoides* gravid females individuals were used in this experiment: 117 *C. circumscriptus* (44.5%), 94 specimens of the Obsoletus complex (35.7%, from which 22 were later identified as *C. obsoletus* based on its progeny); 20 *C. cataneii* (7.6%), 17 *C. newsteadi* (6.5%), 8 *C. paolae* (3%), 5 *C. imicola* (1.9%), 1 *Culicoides puncticollis* (Becker) (0.4%) and 1 *Culicoides jumineri* Callot & Kremer (0.4%).

Culicoides puncticollis and *C. jumineri* were not included in the main results due to the low number of these species and the null survival of its larvae. Although only a single *C. imicola* female oviposited, and developed larvae, results for this species are included due to its epidemiological significance.

6.3.2. Oviposition

The percentage of females that oviposited on the artificial substrate was 35.1%. *Culicoides paolae* showed the highest oviposition rate (62.5%, Fig. 45) followed by *C. circumscriptus* (48.3%). Species from Obsoletus complex, *C. newsteadi*, *C. imicola* and *C. cataneii* had a lower oviposition rate with values of 23.4%, 23.5%, 20% and 15% respectively. As mentioned above, only a single *C. imicola* female oviposited.

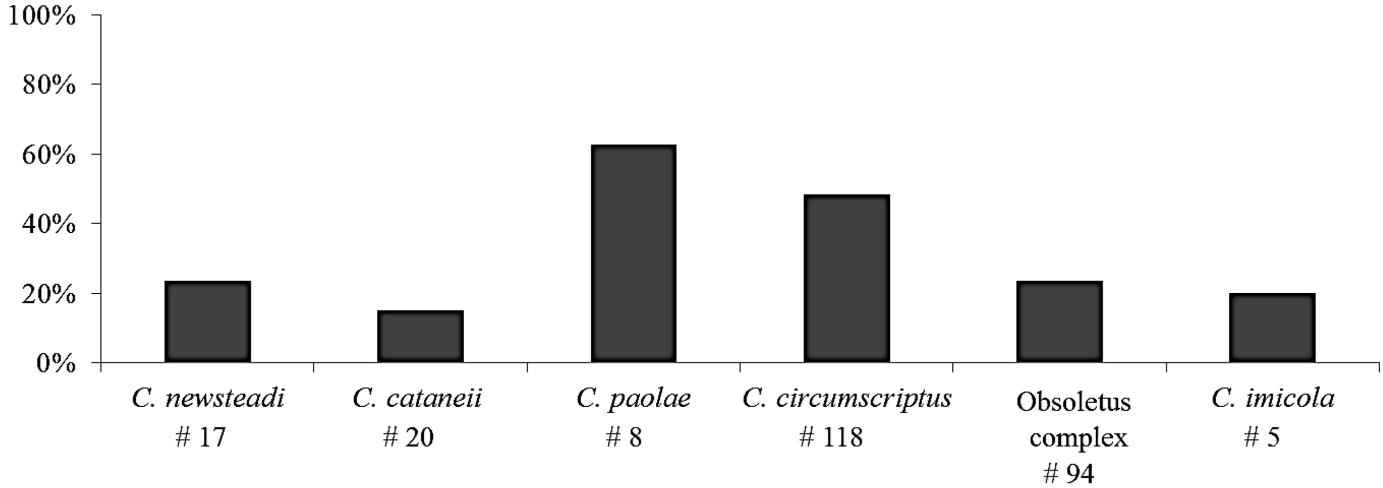


Figure 45. Percentage of field collected, gravid *Culicoides* females that oviposited. (#): Sample size.

The single *C. imicola* female showed the longest oviposition period, being 8 days from collecting females from the field until successful oviposition (Fig. 46). Conversely, *C. newsteadi* showed the shortest period for oviposition (1.5 ± 0.6 days).

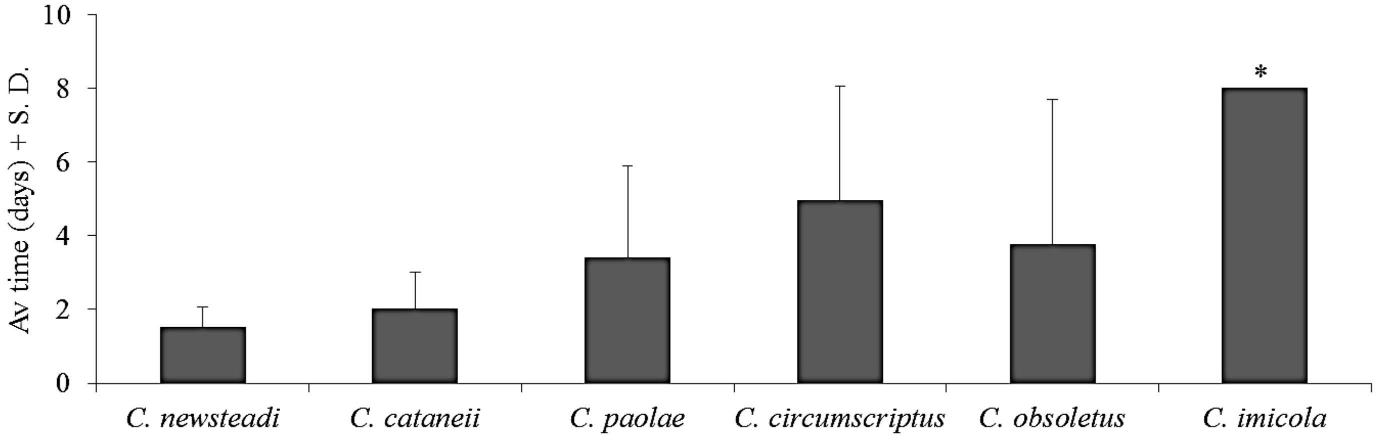


Figure 46. Average (Av) time ± S. D. (Standard Deviation) to oviposit of each field-collected, gravid *Culicoides* females. (*): Based on eggs and larvae of a single individual of *C. imicola.*

The size of the egg batch was similar between species but different to that of previous studies (Table 15). There are no previous studies for *C. paolae*, so, results were compared with that of taxonomically closely related species from the Schultzei complex. The number of eggs laid varied from 54.4 ± 18.3 eggs in case of *C. paolae* to 74 ± 42.7 eggs for *C. cataneii*, except for *C. circumscriptus* which recorded the highest number of eggs oviposited (148.7 ± 73.8 eggs).

Table 15. Range in size of egg batch for various *Culicoides* species. SA: South Africa

	Hill 1947 (1)	**Parker 1950** (1+3)	**Becker 1960** (3)	**Jamnback 1961** (1)	**Nevill 1967** (2)	**Braverman & Linley 1994** (4)	**Veronesi *et al*. 2009** (1)	**Current study** (3)
Country	UK	UK	UK	USA	SA	Israel	SA	Spain
C. newsteadi	-	-	-	-	-	163	-	67.1 ± 16.8
C. cataneii	-	-	-	-	-	126.8 - 157.9	-	74.0 ± 42.7
C. paolae	-	-	-	-	92#	84.5 - 96#	-	54.4 ± 18.3
C. circumscriptus			252	-	-	176 - 215.5	-	148.7 ± 73.8
C. obsoletus	75	30 - 55	-	14.7	-	62 - 74	-	55.6 ± 6.7
C. imicola	-	-	-	-	69	53 - 67	6.8	69*

(*): based on only one individual. (#): Results from Schultzei complex species. (1): Artificial blood feeding, (2): Feeding on an animal host, (3): Natural oviposition of field gravid females, (4): Dissection of gravid females.

6.3.3. Survival of field collected females

The average total lifespan of the total *Culicoides* gravid females was 5.9 ± 4.2 days. *Culicoides imicola* and Obsoletus complex species followed by *C. circumscriptus* showed the longest average lifespan ranging between 5.7 ± 3.2 and 6.8 ± 2.7 days (Fig. 47). Survival of *C. cataneii* females was less than 4 days and represented the shortest lifespan. For all the species, the average lifespan after oviposition was less than three days.

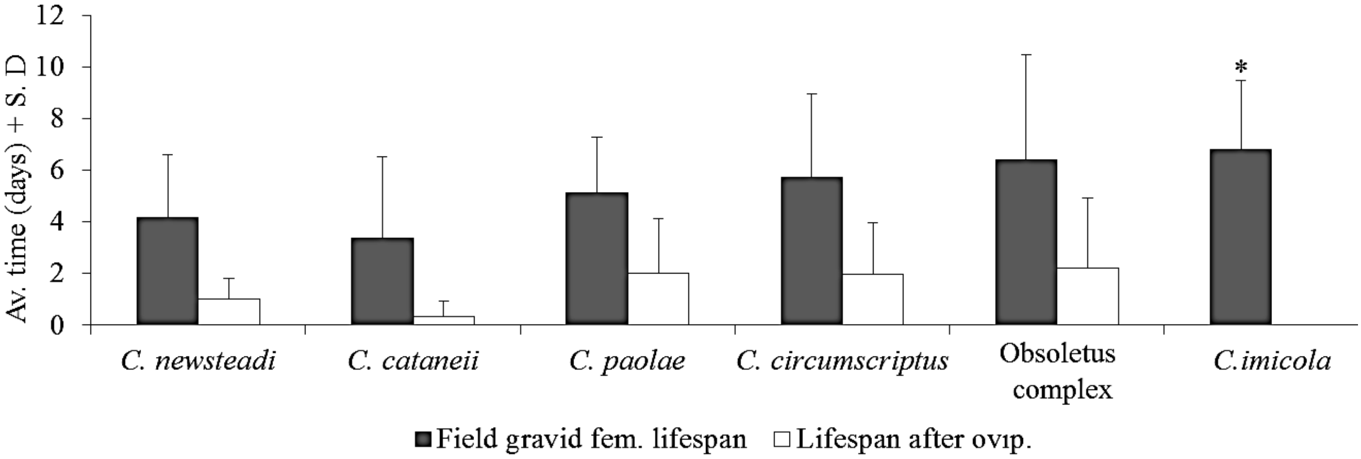


Figure 47. Average lifespan ± S. D. of field-collected *Culicoide*s females and survival after oviposition. (*): *C. imicola* individual died immediately after oviposition.

6.3.4. Life-cycle and F1 adult lifespan

The development time (days) from egg to adult for all species was 28.7 ± 7.2 days on average (Table 16). *Culicoides newsteadi* showed the shortest development period with a mean of 22.5 days. *Culicoides cataneii* followed by *C. obsoletus* showed the longest life-cycle due to longer L4 larvae development; 17.2 ± 7.9 days for *C. cataneii* (40.6% of the development time) and 11.0 ± 7.0 days (39.4%) for *C. obsoletus* (Fig. 48, Table 15). The longest lifespan for F1 adults was reported for *C. imicola* which survived an average of 9.3 ± 0.4 days (36.3% of its life-cycle) followed by *C. paolae* and *C. circumscriptus*.

Table 16. Average duration (days) ± S.D. of the life stages (egg, larva, pupa, F1 adults) of individual *Culicoides* species obtained from field gravid females reared under laboratory conditions.

Species	Egg	L1	L2	L3	L4	Pupa	F1 lifespan	Total cycle
C. newsteadi	2.8 ± 0.5	1.4 ± 0.7	1.3 ± 0.6	2.3 ± 1.2	8.8 ± 2.9	3.3 ± 0.6	2.8 ± 1.3	22.5
C. cataneii	3.8 ± 1.0	7.3 ± 0.6	3.0 ± 1.7	3.7 ± 0.6	17.2 ± 7.9	3.7 ± 0.7	3.7 ± 1.1	42.4
C. paolae	2.7 ± 0.4	4.7 ± 1.5	1.7 ± 0.6	1.7 ± 0.6	5.1 ± 2.8	2.9 ± 0.7	7.2 ± 3.5	25.9
C. circumscriptus	2.1 ± 0.6	3.9 ± 0.9	2.2 ± 0.4	2.1 ± 0.7	5.3 ± 3.2	2.7 ± 0.4	6.7 ± 2.7	25.2
C. obsoletus	2.9 ± 0.9	3.4 ± 1.2	2.5 ± 1.4	2.8 ± 0.9	11.0 ± 7.0	3.0 ± 1.0	2.4 ± 1.1	27.9
C. imicola*	2.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	2.0 ± 0.0	3.5 ± 0.7	2.8 ± 0.4	9.3 ± 0.4	25.5
TOTAL	2.5 ± 0.8	4.1 ± 1.2	2.4 ± 0.8	2.4 ± 1.0	6.8 ± 5.3	2.8 ± 0.6	5.4 ± 3.0	28.2 ± 7.2

(*): Based on eggs and larvae of a single *C. imicola.*

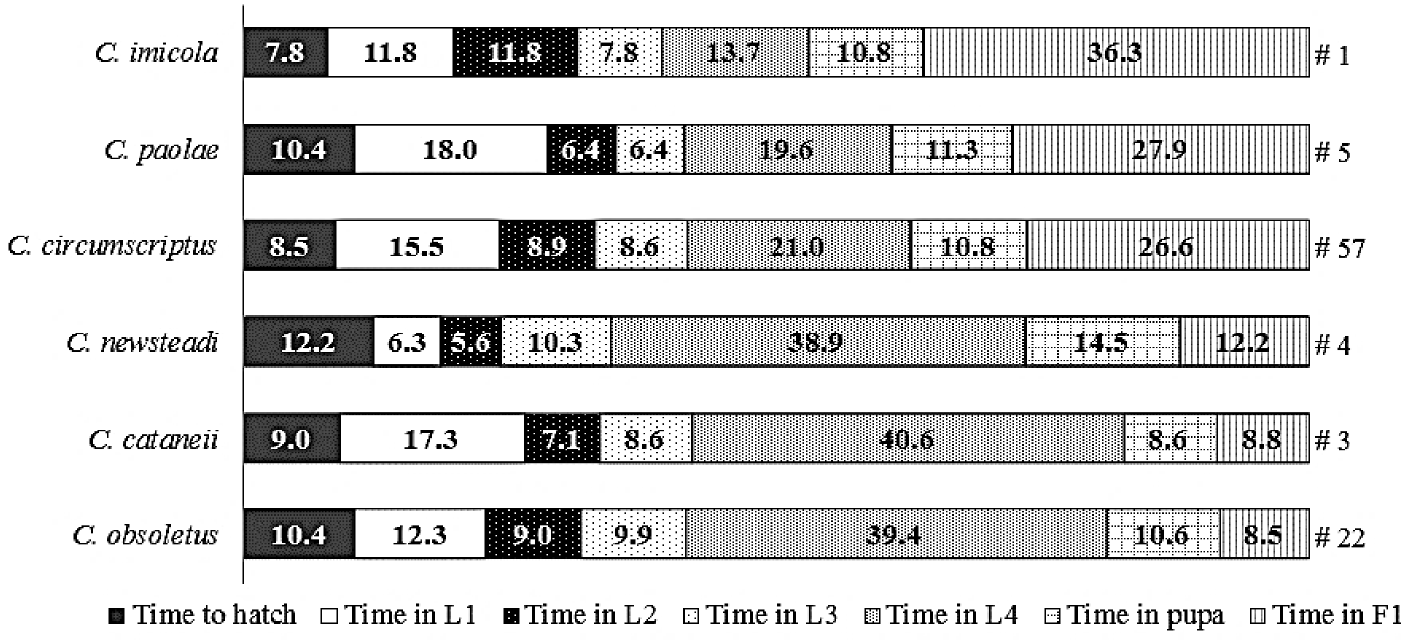


Figure 48. Percentage of sub-adult and adult stages duration, estimated from *Culicoides* field gravid females progeny. (#): Sample size.

6.3.5. Percentage of egg hatching, pupation success and adult emergence

Considering rates of success in passing between different life stages, the highest rates of success were seen at emergence as F1 adult for all the species (89.1 ± 23.0%) followed by the egg hatching (66.8 ± 35.1%) and pupation (47.2 ± 28.4%) (Table 17).

Culicoides imicola followed by *C. newsteadi* showed the highest percentage of survival through egg hatching with 76.8% and 71.6 ± 31.8% of eggs respectively hatching successfully, whereas *C. cataneii* showed the lowest rate of egg hatch (47.4 ± 46.1%). *Culicoides circumscriptus* showed the highest percentage of pupation success (55.4 ± 27.0%); *C. cataneii* and *C. newsteadi* the lowest (11.8 ± 8.7% and 9.2 ± 10.7% respectively). The percentages of adult emergence were equally high for the six species ranging from 68.3 ± 37.3% for *C. newsteadi* to 98.7 ± 4.9% for *C. paolae*.

Table 17. Percentages of egg hatching, larvae pupation and adults emerged for individuals of each *Culicoides* species obtained from field gravid females ± S. D.

	% hatch	**% pupation**	**% emergence**
C. newsteadi	71.6 ± 31.8	9.2 ± 10.7	68.3 ± 37.3
C. cataneii	47.4 ± 46.1	11.8 ± 8.7	90.5 ± 25.6
C. paolae	51.5 ± 34.3	34.5 ± 26.7	98.7 ± 4.9
C. circumscriptus	70.4 ± 33.9	55.4 ± 27.0	91.3 ± 17.1
C. obsoletus	62.3 ± 37.9	41.2 ± 24.8	84.2 ± 32.3
C. imicola	76.8*	26.1*	83.8 ± 2.8
TOTAL	66.8 ± 35.1	47.2 ± 28.4	89.1 ± 23.0

(*): Based on eggs and larvae of a single *C. imicola.*

6.3.6. Percentage of pupation per day

The average of total pupation per day within all the species was 6.0 ± 9.1%. Pupation of *C. circumscriptus* and *C. paolae* were clustered between the 8th and the 17th day after egg hatching (Fig. 49). Larvae of *C. obsoletus*, *C. circumscriptus* and *C. paolae* started to pupate after a similar period (8 days); however, *C. obsoletus* pupation was more evenly distributed and some larvae pupated over a longer period of up to 44 days after egg hatching. In the case of *C. cataneii*, pupation was evenly distributed starting on day 17 and continue until day 47 after egg hatching, thus showing the widest period of pupation among all species.

The shortest period of pupation was obtained for *C. imicola* and *C. newsteadi*, in fact, pupation was concentrated over one to two days. All of *C. imicola* larvae pupated between the 11th and the 12th day after egg hatching, while the pupation of *C. newsteadi* larvae were clustered around the 23rd day after egg hatching.

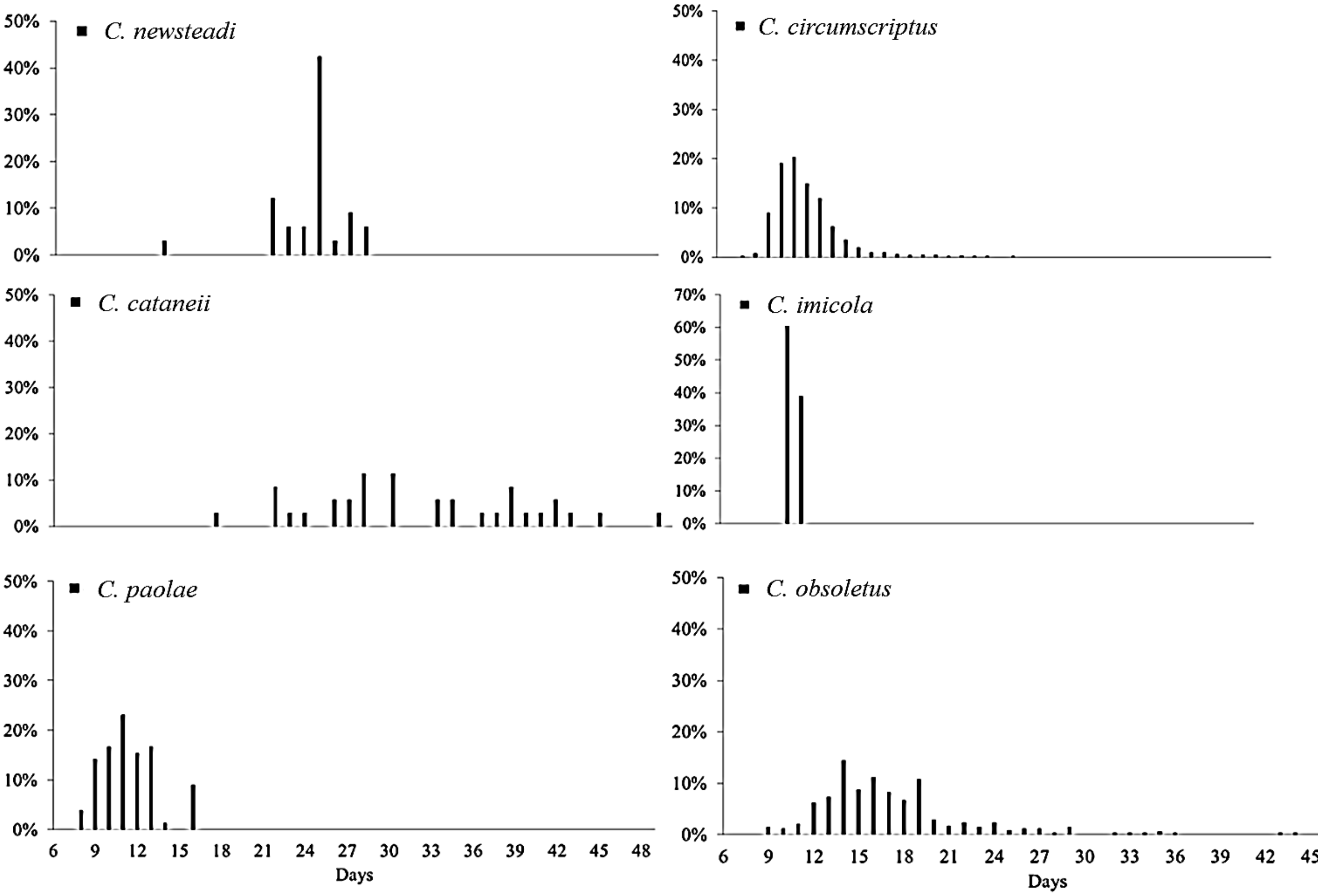


Figure 49. Percentage of larvae that pupated daily, estimated from the progeny of each field collected, gravid *Culicoides* female.

6.3.7. Sex ratio

The sex ratio was male biased for *C. obsoletus*, *C. imicola* and *C. newsteadi*, while it was female biased for *C. cataneii* and *C. paolae* (Fig. 50). Only *C. circumscriptus* showed an equal sex ratio between males (50.7 %) and females (49.3 %).

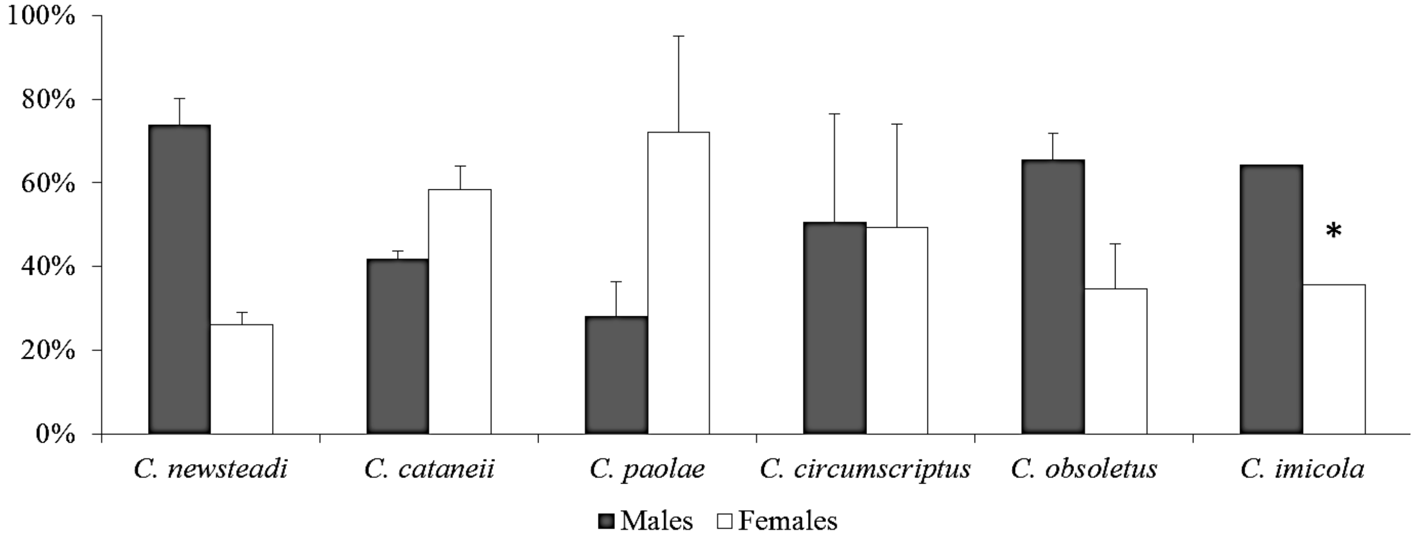


Figure 50. Average percentage ± S. D. of adult males and females emerged in the laboratory by *Culicoides* species emerged in the laboratory (*): *C. imicola* progeny are from only one field-collected gravid female.

Tale 18 summarise the suitability for rearing by species considering all the life history parameters together. We consider that high percentage of oviposition, high number of eggs, long lifespan and high percentage of egg hatch, pupation and F1 adult emergence are indicators of high suitability; whereas long time to oviposit, long life-cycles and sex ratio values higher than 1 are indicators of poor suitability for rearing that species in laboratory conditions. High values in the last column mean high suitability of this species.

Table 18. Summarize of reproductive potential across the parameters measured from each *Culicoides* species. Positive parameters: Parameters that increase the suitability of the species to laboratory conditions. Negative parameters: Parameters that decrease the suitability of the species to laboratory conditions. Sex ratio= 1- (% females/% males). Total values of the last column are the results of the following equation: (positive parameters-negative parameters).

	Positive parameters							**Negative parameters**			
Species	**% Oviposition**	**# eggs**	**Total lifespan (days)**	**Lifespan after ovip. (days)**	**% egg hatch**	**% pupation**	**% F1 adult emerg.**	**Time to oviposit (days)**	**Total life-cycle (days)**	**Sex ratio**	**TOTAL values**
C. newsteadi	23.5	67.1	4.2	1.0	71.6	9.2	68.3	1.5	22.5	0.6	220.3
C. cataneii	15.0	74.0	3.4	0.3	47.4	11.8	90.5	2.0	42.4	-0.4	198.4
C. paolae	62.5	54.4	5.1	2.0	51.5	34.5	98.7	3.4	25.9	-1.6	281
C. circumscriptus	48.3	148.7	5.7	2.0	70.4	55.4	91.3	5.0	25.2	0.0	391.6
Obsoletus complex	23.4	55.6	6.4	2.2	62.3	41.2	84.2	3.8	27.9	0.5	243.1
C. imicola*	20	69	6.8	0	76.8	26.1	83.8	8	25.5	0.4	248.6

6.3.8. Artificial blood feeding for Obsoletus complex NF

A total of 128 Obsoletus complex NF were obtained from field and offered a blood meal using artificial methods. Only 8 *C. obsoletus* individuals managed to feed (6.25%).

The percentage of oviposition of artificially blood fed females was higher than the field gravid females (37.5%, Fig. 51). Artificially blood fed females showed a higher lifespan than the field gravid females while the lifespan after oviposition was similar (Fig. 52). No significant differences were found in the percentage of oviposition and the lifespan of artificially fed and field gravid females (2-tailed K-S-test, *P*>0.05).

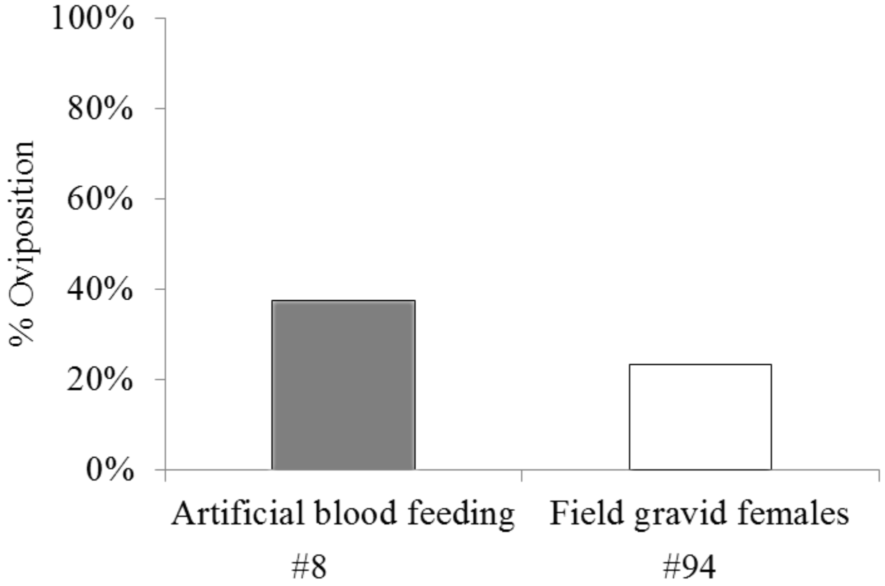


Figure 51. Percentage of oviposition of field-collected Obsoletus complex nulliparous females fed through artificial membrane versus oviposition of field-collected, gravid Obsoletus complex females. (#): Sample size.

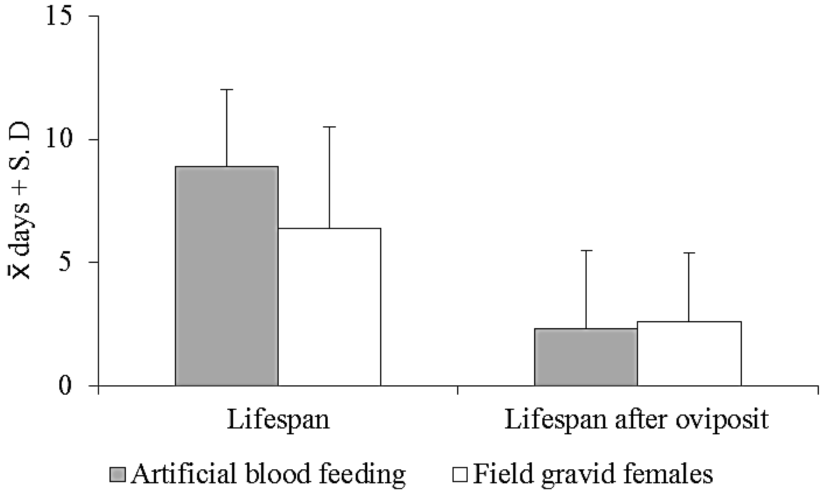


Figure 52. Average ± S. D. lifespan of field-collected *C. obsoletus* nulliparous females fed through artificial membrane after feeding versus average lifespan of field-collected, gravid *C. obsoletus* females and survival after oviposition.

Time to egg hatching, time to adult emergence, and the lifespan of the F1 adults were higher on artificial blood feeding individuals whereas the time to oviposit and the larval development were higher on *C. obsoletus* field gravid females (Fig. 53), however, no significant differences were found within the artificially fed and the field gravid females (2-tailed K-S-test, *P*>0.05).

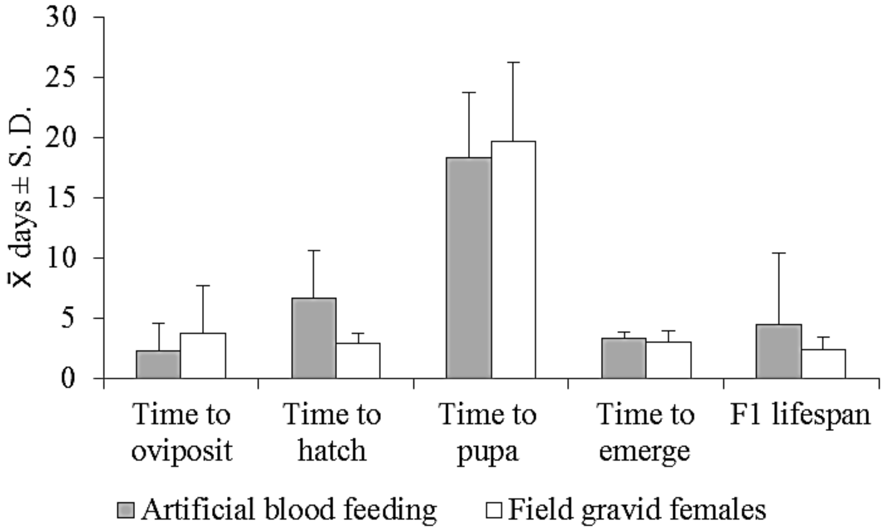


Figure 53. Average time ± S. D. to oviposit, to egg hatch, larval period and time to adult emergence of field-collected *C. obsoletus* nulliparous females fed through artificial membrane versus field-collected, gravid *C. obsoletus* females.

The progeny from *C. obsoletus* field gravid females showed higher percentage of females than the ones fed with artificial membrane (Fig. 54). The average number of eggs, pupae and adults of the F1 were higher on *C. obsoletus* field gravid females (Fig. 55). No significant differences were found in that results from engorged and field gravid females (2-tailed K-S-test, *P*>0.05).

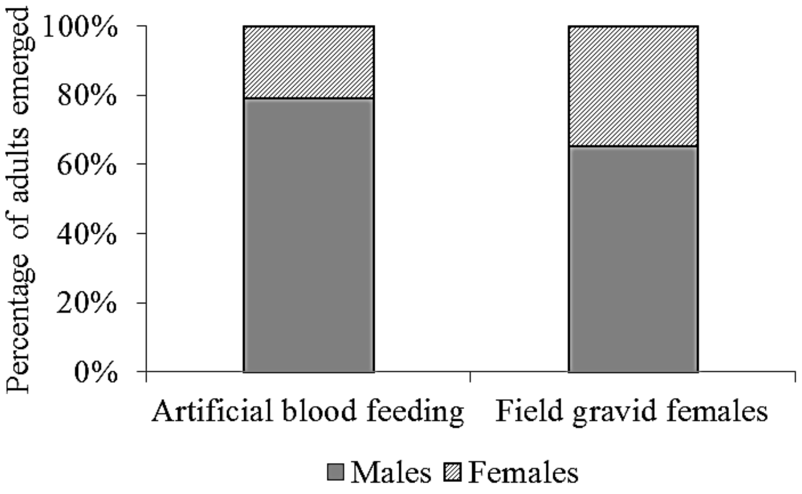


Figure 54. Percentage of F1 males and females emerged in the laboratory from *C. obsoletus* artificially fed and *C. obsoletus* field gravid females.

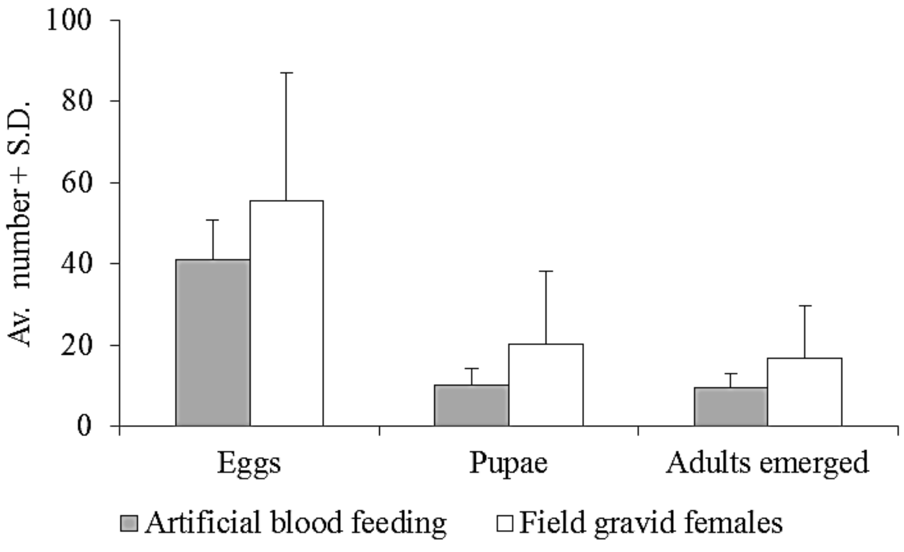


Figure 55. Average number ± S. D. of eggs, pupae and adults obtained from field-collected *C. obsoletus* nulliparous females fed through artificial membrane versus field-collected, gravid *C. obsoletus* females.

The percentage of egg hatching and the F1 adults emergence were similar on artificial blood fed individuals whereas the percentage of pupation was higher on field gravid females (Table 19). However, no significant differences in these bionomic parameters were found between artificial engorged and field gravid females (2-tailed K-S-test, $P>0.05$).

Table 19. Percentage of egg hatching, larvae pupation and F1 adults emerged from *C. obsoletus* individuals artificially fed and *C. obsoletus* field gravid females ± S. D.

	% hatch	**% pupation**	**% emergence**
Artificial blood feeding	63.5 ± 26.3	14.5 ± 14.4	90.0 ± 10.1
Field gravid females	62.3 ± 37.9	41.2 ± 24.8	84.2 ± 32.3

6.4. Discussion

6.4.1. Species composition

The species composition determined in this study with OVI traps were similar to that of previous studies in Majorca Island (Del Río *et al*. 2013, 2014a, 2014b). The major vector species *C. imicola* and *C. obsoletus,* as well as the potential vector *C. newsteadi,* are well established in the Balearic Islands (Miranda *et al*. 2003, 2004). Although captures of *Culicoides* gravid females were low, UV light traps are still considered the most reliable and generally effective method for capturing large quantities of adult female biting midges including gravid individuals (Viennet *et al*. 2011). In fact, we obtained a high proportion of gravid females of ornithophilic species such as *C. circumscriptus* (Pettersson *et al*. 2013, Bobeva *et al*. 2015, Martínez-de la Puente *et al*. 2015); comprising the 44.5% of the total gravid females collected from the field and being more abundant than previous studies conducted in the Balearics (Miranda *et al*. 2003, 2004, Del Río *et al*. 2013). Gravid females of *C. circumscriptus* were commonly more abundant in UV light trap samples than that of mammophilic species. The reason for this phenomenon remains mostly unexplained but may be linked with longer gonotrophic cycles (Carpenter *et al*. 2006b). The other ornithophilic species captured in this study was *C. paolae*, which is present in Spain (Estrada *et al*. 2011) and has been recently implicated in the transmission of avian haemosporidians (Veiga 2018). Taxonomically, *C. paolae* was first included in the Old World Schultzei Complex (=subgenus Remmia Glukhova, 1977) due to its taxonomical and biological similarities. Later, it was repositioned by Meiswinkel *et al*. (2004b) in the Copiosus group (Vargas, 1960), however, the placement of this species still unclear (Augot *et al*. 2017b). This species is suspected to breed in fruits of plants from genus *Opuntia* sp. which is an invasive species widely distributed in the Mediterranean countries (Meiswinkel *et al*. 2004b).

6.4.2. Oviposition

The overall oviposition rate (35.1%) of field collected *Culicoides* females on the artificial substrate indicates that the method used in the current study was adequate to obtain eggs. However, the method could be improved since half of the gravid females did not lay eggs. The reasons for the lack of oviposition of certain species are still unknown. The study of Carpenter *et al*. (2001) described a re-absorption of eggs in *C. impunctatus* species when substrate was not optimal and highlighted that the use of oviposition substrates that better reflect natural oviposition habitat of species could improve the oviposition rates (e.g. for *C. impunctatus*, infusing the upper layers of substrates with *Sphagnum* sp.). *Culicoides* individuals were kept in dark during the major period of the current experiment. This fact may be affected the photoperiod of these species. Although prior studies suggested that abundance and development of Obsoletus complex species were not affected by photoperiod (Searle *et al*. 2012, Lühken *et al*. 2015), species such as *C. pulicaris* and *C. impunctatus* showed earlier peaks of adult abundance when the number of daylight hours increase.

Culicoides paolae and *C. circumscriptus* females showed a high percentage of oviposition, similar to the 36% - 54% recorded by Fahrner & Barthelmess (1988) for *C. nubeculosus* under laboratory conditions. Additionally, Obsoletus complex females showed a higher oviposition rate than of 7% recorded by Jamnback (1961) in the UK. Hence, high oviposition rates of those species could be linked to its capacity to adapt to different substrates, including artificial ones, being in special interest for laboratory rearing (Kettle and Lawson 1952, Mellor and Pitzolis 1979, Harrup *et al*. 2013, Zimmer *et al*. 2013a, 2014b, Steinke *et al*. 2016, Braverman *et al*. 2018).

Only one female of *C. imicola* succeed in ovipositing after almost 8 days, this is twice as long as the time obtained by Veronesi *et al*. (2009) at the same temperature (25ºC). This result along with the low numbers collected, highlights the difficulties in rearing *C. imicola* under laboratory conditions.

Eggs of *C. obsoletus* were laid earlier compared to the results of Hill (1947), Parker (1950) and Jamnback (1961), where oviposition took place between the first and the second week after the blood meal intake. This fact may be related to the artificial blood feeding used in other studies versus the field blood feeding of the present study and the lower temperatures at which *C. obsoletus* were maintained. Conversely, the average oviposition time of *C. circumscriptus* was similar to that of Becker (1960) who recorded an average oviposition time of 4.5 days from field gravid females.

Culicoides circumscriptus laid the highest number of eggs among all species in this study, which is probably linked to its largest body size (2.2 mm; Delécolle, 1985). This species could laid eggs more than one time during the current experiment due to its autogenous nature (Kettle 1977); however, the average number of eggs laid by this species along with *C. paolae, C. newsteadi* and *C. cataneii* were lower than that reported previously (Becker 1960, Nevill 1967, Braverman and Linley 1994). These fecundity differences have no clear explanation. The aforementioned egg absorption and bionomic particularities related to local populations or effect of the artificial rearing conditions (i.e.: temperature, humidity and feeding diet) could be contributing factors.

The number of eggs oviposited by *C. obsoletus* and *C. imicola* were similar to previous studies (Hill 1947, Parker 1950, Nevill 1967, Braverman and Linley 1994) excepting Jamnback (1961) and Veronesi *et al*. (2009), that reported lower number of eggs per female than our results and the above mentioned studies. Results obtained from a single *C. imicola* female prevent us to make conclusions about factors affecting fecundity of this species. Prior studies have found influence of artificial versus natural blood feeding; as well as the age of the field collected females on fecundity (Nevill 1967, Braverman and Linley 1994, Veronesi *et al*. 2009).

6.4.3. Survival of field collected females

Females of the vector species *C. imicola* and *C. obsoletus* showed the longest lifespan after field collection. The age of the field collected females could not be assessed but adult *Culicoides* species can survive for about 20 to 30 days (Mellor 2000, Purse *et al*. 2015) and exceptionally up to 92 days (Goffredo *et al*. 2004). If we assume that *C. imicola* and *C. obsoletus* have a longer lifespan than the other species captured, this result would be of epidemiological importance since female survival increases the vector capacity of a species (Mullens *et al*. 2004). However, our recorded lifespan for *C. obsoletus* was notably lower than that obtained by Parker (1950), Jamnback (1961) and Goffredo *et al*. (2004), where individuals from the Obsoletus complex survived at least 40 days fed with sugar solution. We used field collected gravid females with unknown age and number of gonotrophic cycles, whereas the aforementioned studies used mainly NF that are younger and expected to live longer. Other possible explanation could be related with laboratory temperature. Lower temperatures increase the lifespan of *Culicoides* females (Gerry and Mullens 2000, Mullens *et al*. 2004), therefore, longer lifespan values recorded by Parker (1950), Jamnback (1961) and Goffredo *et al*. (2004) may be related with the temperatures where NF were kept (13-23°C), being lower than the 25ºC of the current study.

Similar to the observations of Hill (1947), Nevill (1967) and Becker (1960), *Culicoides* collected in the present study died immediately or within two days after egg laying. This was an unexpected result since anautogenous females (e. g. *C. obsoletus* and *C. imicola*) and autogenous females (e. g. *C. circumscriptus*) should survive a minimum of two and three gonotrophic cycles respectively (Linley 1983, Purse *et al*. 2015). Our results may be explained by the age of the field gravid females collected in the current study (being probably “old” females in its second/third gonotrophic cycle) or the unsuitable laboratory conditions for these species.

6.4.4. Life-cycle and F1 adult lifespan

The short life-cycle of the ornithophilic species *C. paolae* and *C. circumscriptus* linked to its high oviposition rates and relative long F1 lifespan, render this species candidates for laboratory rearing. Conversely, the long life-cycles recorded for *C. cataneii* and *C. obsoletus* due to its long L4 larva stage indicate that it will be difficult to rear these species in the laboratory. The agar may have a deterrent effect on larvae and pupae of species of the Obsoletus complex, which breed in soil with higher concentration of lignin and fibre (Zimmer *et al*. 2013a) and also prefer dry and non-flooded substrate (Lühken *et al*. 2014b). In addition, *C. cataneii* breeds in vegetated pool shorelines and avian habitats which suggest it to feed on birds (Boorman 1974, Foxi and Delrio 2010, Talavera *et al*. 2015, 2017b). It was aforementioned that photoperiod could also affect the development of these species since the individuals were kept in dark. Hence, different moisture regimes and photoperiods as described by Lühken (2014b, 2015) can be used to improve the pupation rate of *C. obsoletus* and *C. cataneii*.

This is the first study that has obtained a F1 adult of the vector species *C. obsoletus*, *C. newsteadi* and the non-vector species *C. cataneii* and *C. paolae*, and also of Mediterranean populations of *C. circumscriptus* and *C. imicola*, highlighting the novelty of this study. *Culicoides newsteadi* showed a short lifespan for both field gravid females and for F1 adults, as well as low oviposition rates. These results mean that this vector species is likely to be difficult to rear in the laboratory. *Culicoides imicola* females obtained from a single specimen showed the longest lifespan; however, its lifespan was lower in comparison to that of Veronesi *et al*. (2009) where adults emerged also at 25ºC survived almost 18 days. This discrepancy may be related with the aforementioned age of female individuals used in either studies or also the biological differences between the population of *C. imicola* from South African used in Veronesi *et al*. (2009) and the population from the Balearic Island used in the current study.

Since biting midges spend half of their life cycle as sub-adults, it is very important to define the drivers that modulate pre-imaginal development to determine if the sub-stages are vulnerable to control measures or changes in abiotic conditions. In fact, the pre-adult development determines the peaks and the number of generations per year of species such as Obsoletus complex (White *et al*. 2017). To date, research has focused on adult control and field knowledge on the factors regulating sub-adult stages remains mostly unexplored (Harrup *et al*. 2016). Furthermore, control strategies should focus on reducing the first peaks of bivoltine species since the immature stages are released from density-dependence regulation (White *et al*. 2017).

6.4.5. Percentage of egg hatching, pupation success and adult emerge

The average range of egg hatching among all *Culicoides* species agrees with that of the reference colonies of *C. nubeculosus* under laboratory conditions (57.1 – 69.1%; Fahrner and Barthelmess 1988).

The percentage of egg hatching, pupation and adults emerged for *C. imicola* were similar to that of Veronesi *et al*. (2009), showing that pupation would be one of the critical factors for *C. imicola* rearing, probably related with its preference to relative dry habitats for pupation. In fact, *C. imicola* pupae are not able to float to the surface (Nevill 1967) and adults may have difficulties in emerging from a wet substrate, explaining the lower percentage of adult’s emergence when compare with the other species.

Culicoides cataneii showed the lowest percentage of egg hatching and percentage of pupation, possibly related to its biology or even the difficulties discussed above about the non-suitable substrate to lay eggs and pupate.

In spite of its high percentage of egg hatching and short life-cycle, *C. newsteadi* showed rearing limitations under artificial conditions due to its low pupation rate and adult emergence. This was unexpected since *C. newsteadi* was considered a promising candidate for laboratory rearing, being very abundant at farm level and a potential vector of BTV (Goffredo *et al*. 2015), This species is actually considered a cryptic species included in the Newsteadi complex (Pagès *et al*. 2009); with probably bioecological differences among them; so, environmental requirements of members of this complex must be investigated in order to understand whether they differ in bionomics.

Culicoides circumscriptus showed a lower average of eggs hatching in the current study than that indicated by Becker (1960). However, Becker (1960) obtained lower percentage of emerged adults than the present work. This discrepancy could be attributed to the wide range of temperatures used in the aforementioned work (14.5-27.8°C) compared to the present work (25°C), or even the above mentioned effect of photoperiod on *C. circumscriptus*. Individuals in Becker (1960) were kept at 12:12 h photoperiod, whereas the ones in the current study were maintained most of the experiment in dark.

According to these results, pupation rate could be considered the most critical step in *Culicoides* rearing under laboratory conditions. A possible explanation (apart of the limitation of ‘artificial’ breeding sites) could be competition for resources or even cannibalism that was recorded in the current trials (larvae being predatory on the pupa) reducing the survival rate of species. This behaviour was also recorded by Nevill (1967) and commented in Veronesi *et al*. (2009). The substrate could also influence over this parameter; in fact, Harsha and Mazumdar (2015) observed a higher survival rate of *C. peregrinus* larvae reared using liquid/cotton wool instead of the semi-solid agar substrate used in the current study.

Conversely, the percentage of adults emerged from reared pupae was relatively high for all of the species assayed; therefore, although the use of artificial substrates, adult emergence seems a less-critical step during the life-cycle of *Culicoides* under laboratory conditions than pupation from L4 larvae.

6.4.6. Percentage of pupation per day

The pupation period for *C. circumscriptus, C. paolae* and *C. imicola* were concentrated in 2-8 days similar to the results of Becker (1960) and Veronesi *et al*. (2009). For *C. obsoletus, C. cataneii* and *C. newsteadi* the pupation was later and L4 larvae stage had longer duration; so, pupation occurred over a long period and was not synchronised over a short time as described for *C. circumscriptus*, *C. paolae* and *C. imicola*. This fact may be related with different responses to temperature variation and other biotic and abiotic factors among the species.

6.4.7. Sex ratio

Males were predominant except for *C. cataneii* and *C. paolae*, which had a higher proportion of females. *Culicoides circumscriptus* had a similar percentage of males and females supporting the results from Becker (1960) who obtained an average of 51% females. On the contrary, *C. newsteadi*, *C. obsoletus* and *C. imicola* showed lower ratio of females to males emerging from pupae. The reason for this remains unclear, higher mortality among female larvae or sex determination based on the temperature, as well as limitations of the rearing diet for female development, are possible explanations (Nevill 1967, Veronesi *et al*. 2009).

6.4.8. Artificial blood feeding for Obsoletus complex NF

The percentage of artificially blood fed females was low and the bionomic results of this study were obtained from only 8 *C. obsoletus* engorged females; so, these data should be taken as a preliminary and qualitative indication of the potential of the feeding technique.

The percentage of total *C. obsoletus* engorged females obtained on artificial membrane (6.25%) agrees with the 6.21% obtained by that of Goffredo *et al*. (2004) and more than the 5% recorded by that of Barber *et al*. (2018) using the same artificial membrane. Innovate feeding techniques have improved the percentage of blood feeding in prior studies, such as use of different types of membranes like chicken skin, changing the feeding orientation on the Hemotek (facing up or facing down), changing the time of day of feeding or the blood temperature recorded (de Beer *et al*. 2018).

Culicoides obsoletus NF fed upon artificial membrane and *C. obsoletus* field-collected gravid females showed high oviposition rate (37.6% and 23.4% respectively). These values were higher than the ones recorded by Jamnback (1961) in the UK, indicating that the system we used was more appropriate for oviposition. In fact, Jamnback (1961) kept the insects individually in cotton-stoppered four-dram vials with a piece of moist cotton in the bottom of each vial as oviposition substrate, whereas in our study oviposition substrate consisted of filter paper and moistened cotton in cardboard boxes (Fig. 36).

Artificially blood fed females exhibited higher lifespan than the gravid ones, obviously because NF used for artificial feeding were younger on average and lived longer than the gravid ones. However, our recorded lifespan for *C. obsoletus* was still lower than the obtained in other studies such as Parker (1950), Jamnback (1961) and Goffredo *et al*. (2004), where individuals of *C. obsoletus* survived more than 40 days maintained on a sugar solution. Isolation of the individuals, size and material of containers, as well as laboratory conditions could affect the survival rates of these species. In the current study we used cardboard containers whereas Parker (1950), Jamnback (1961) and Goffredo *et al*. (2004) used glass or plastic containers for the NF. In addition, Jamnback (1961) kept a single NF in each container as in the current study, while Parker (1950) and Goffredo *et al*. (2004) keep more than one female together. Parker (1950), Jamnback (1961) and Goffredo *et al*. (2004) kept the NF at similar or lower temperature than the 25ºC of the current study. It was previously found that lower temperatures increase lifespan of *Culicoides* (Gerry and Mullens 2000, Mullens *et al*. 2004) and this could explain the longer lifespans found in the above mentioned studies.

In agreement to Hill (1947), Nevill (1967) and Becker (1960), *Culicoides* died immediately or within two days after oviposition. This was an unexpected result since *C. obsoletus* is considered an anautogenous species and can survive a minimum of two gonotrophic cycles (Linley 1983, Purse *et al*. 2015). In fact, Parker (1950) observed that two *C. obsoletus* females accepted second blood meals after oviposition and laid second egg batches. As previously commented, we do not know the age of the field caught individuals; so, we cannot demonstrate that the low lifespan after oviposition was related to the non-suitable laboratory conditions for this species.

Oviposition was earlier in artificially engorged females (≈ 2 days) compared to the results of Hill (1947), Parker (1950) and Jamnback (1961), where oviposition took place between the first and the second week after blood feeding. This difference may be related to the artificial blood feeding technique since the prior studies fed females using cotton wool dipped in blood. This technique could decrease blood quality due to its desiccation. In addition, artificially engorged females in Hill (1947), Parker (1950) and Jamnback (1961) were maintained at different, often lower temperatures (13-23°C), that could also increase the time for eggs maturation. Another possible explanation for the longer time to oviposition could be the host blood source. The above mentioned studies used human or rabbit blood, whereas we used cattle blood since *C. obsoletus* have preference for this host (Martínez-de la Puente *et al*. 2012, Pettersson *et al*. 2013, Elbers *et al*. 2018).

Artificially blood fed females showed lower time to oviposit than the field gravid females. This is an unexpected result; blood fed females need time for the development and maturation of the eggs, thus increasing the time to oviposit, whereas eggs are already mature in gravid females. This fact could be related with the daily variation of field temperatures (i. e. 31.2 ºC max. - 18.3ºC min. in July, AEMET 2019) versus the constant temperature in the laboratory, accelerating the oviposition cycles of *Culicoides* females. Other explanation could be the period that the NF had spent in the laboratory before the blood feeding (3 days). During this period, the future blood fed females could be acclimatised earlier to the laboratory conditions than the field gravid females.

The number of eggs oviposited by artificially engorged *C. obsoletus* females was similar to Parker (1950); higher than Jamnback (1961) and lower than Hill (1947) using artificial blood feeding methods. These results were unexpected since these studies used blood from humans or rabbits which are not the main hosts for of *C. obsoletus*. Possible explanations for this variation could be the aforementioned *Culicoides* oviposition cycles conditioned by temperature or also the oviposition substrates that could made a deterrent effect on the oviposition process.

The number of eggs laid, number of pupae, percentage of pupation, number of total F1 adults emerged and percentage of F1 females emerged by field collected gravid females was higher than the artificially engorged females. These results may be explained by the following reasons: the blood source, being better the one obtained during the natural field feeding activity of the *C. obsoletus* females. The blood from the Hemotek was previously defibrinated, which could decrease its quality. In fact, artificial blood feeding could decrease the reproductive potential of *Culicoides* individuals (Carpenter *et al*. 2001, Veronesi *et al*. 2009) and studies such as Fahrner and Barthelmess (2018) demonstrated that *C. nubeculosus* laid more eggs using fresh blood. It was also aforementioned that insects in field conditions are subjected to diurnal variation in temperature. Temperature accumulation may affect the reproduction metabolism of *Culicoides* females (Lühken *et al*. 2015), however, the individuals kept in the laboratory were constantly maintained at the same temperature. Future experiments with higher number of engorged individuals and variations of temperatures in the laboratory should be considered in order to confirm our preliminary results.

Conversely, the percentage of total egg hatching and F1 adult emergence was higher in *C. obsoletus* fed upon artificial membrane. These results were unexpected since we were used the individuals from the same population. In fact, Fahrner and Barthelmess (2018) recorded higher egg hatching and adults emerged using fresh blood.

To sum up, although no statistically significant differences were found between bionomics of artificial engorged females and field gravid females; parameters such as percentage of oviposition, percentage of adults emerged and adults lifespan were as high in the results obtained from the artificially engorged females; demonstrating the validity of the blood feeding method. Meanwhile, the number of total eggs, pupae, adults emerged and percentage of pupation was higher in field collected gravid females. An increase in the sample size of engorged females is needed in further experiments to demonstrate significant differences in bionomic parameters between artificial engorged and field gravid females.

These results suggest that variation in bionomics may be related to many different factors including feeding methods, type and condition of blood employed and also maintenance conditions like temperature regimes and oviposition substrates. In fact, we are still far away to fully understand the conditions in which to keep European *Culicoides* BTV vector species to allow consistent laboratory rearing. For example, one of the knowledge gaps is the mating conditions required by different *Culicoides* species (Jamnback 1961, Kettle 1977, Veronesi *et al*. 2009). It is known that *Culicoides* mate in swarms (Kettle 1995, Kirkeby 2018), however, the details of this behaviour in vector *Culicoides* species remains unknown (Blackwell *et al*. 1992, González *et al*. 2017). Future studies covering these aspects should be undertaken.

6.5. Conclusions

This work contributed to the knowledge of the basic bionomics of *Culicoides* species associated with farm animals in Europe. A novel methodology for obtaining viable eggs from field-collected females is described and the life-cycle for six *Culicoides* species was completed under laboratory conditions and compared in terms of the duration and success rates of different life-history stages.

Overall our results suggest that *Culicoides* species are particularly sensitive in larvae stage of their life-cycle and least sensitive in pupa stage, but this needs to be verified with empirical data from more individuals and species. Target control measures at the L4 stage should be considered since species tend to spend most time in it and natural survival rates are low.

According to the data from this study, the ornithophilic species *C. paolae* and *C. circumscriptus* are the most promising species to be reared in laboratory conditions due to its high oviposition rates, F1 lifespan and sex ratio, meanwhile, the major vector species *C. obsoletus* and *C. imicola* showed important limitations of low success of oviposition and other species such as *C. cataneii*, and *C. newsteadi* had difficulties to pupate successfully in the artificial substrate.

Finally, further research evaluating different oviposition substrates and temperatures for larvae incubation (Becker 1960) deserves attention, including different diets for both adults and larvae (Harsha and Mazumdar 2015, Erram and Burkett-Cadena 2018) as well as recording of development at different temperatures.

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7. Study of the Obsoletus complex bionomics and other livestock associated biting midges *Culicoides* at different temperatures in laboratory conditions




































7. Study of the Obsoletus complex bionomics and other livestock associated biting midges *Culicoides* at different temperatures in laboratory conditions

7.1. Introduction

Culicoides biting midges can transmit globally important viral pathogens of domestic livestock such as BTV that causes an infectious, non-contagious of disease of ruminants known as bluetongue (Mellor *et al*. 2000). Temperature and humidity are the major environmental factors affecting development, activity and distribution of *Culicoides* populations in Europe (Murray 1991, Wittmann *et al*. 2002, Carpenter *et al*. 2008b). In fact, adult activity and reproduction occur year round in tropical areas (Sellers 1980, Verhoef *et al*. 2014), but in temperate and cool regions they overwinter as larvae and some adult species enter in diapause when daylight hours and/or temperature declines below a threshold level (Kettle 1962, Rawlings and Mellor 1994, Lysyk and Danyk 2007). Moreover, the length of the adult vector-free period (SVFP) determines whether BTV will persist between years (EFSA 2017).

Several studies in laboratory conditions, such as Veronesi *et al*. (2009) revealed that temperature affects the development of sub-adult stages of *C. imicola*. Also, in case of Obsoletus complex species, temperature and composition of the breeding substrate plays an important role in larvae development (Lühken *et al*. 2014a, 2015, Steinke *et al*. 2015). Other studies showed the effects of temperature on early stages, adult size and seasonality on *C. sonorensis* (Akey *et al*. 1978, Lysyk and Danyk 2007, McDermott *et al*. 2016); *C. arakawae* and *C. maculatus* (Kitaoka 1982); as well as *C. imicola* and *C. bolitinos* (Mullens 1987, Verhoef *et al*. 2014).

The study of Purse *et al*. (2015) summarized the data available about the relationship between temperature and *Culicoides* development. This study showed that development time of *Culicoides* immature stages increased with temperature but percentages of survival were different within species. However, data in Purse *et al*. (2015) put on evidence the little information available regarding the effect of temperature on the most common European species. In fact, only five species were included in that study (*C. sonorensis*, *C. nubeculosus*, *Culicoides variipennis* (Coq.), *C. brevitarsis* and *C. imicola*).

The present study shows detailed information about the basic bionomics of two different *Culicoides* populations reared at different temperatures in laboratory conditions. Except *C.*

imicola, this is the first study comparing the effect of temperatures among all the stages of the entire *Culicoides* life-cycle from two populations of field gravid females. In fact, results obtained in this study included information from species never reared before in a laboratory, such as *C. paolae*.

7.2. Material and Methods

Adult *Culicoides* were collected alive from two cattle farms located in Majorca (Balearic Islands, Spain) separated 55 km from each other (Fig. 56); one in Felanitx municipality (*Can Cosme*, 39° 31' 21.12"N; 3° 6' 21.55"E) and the other in Palma municipality (*Son Ajaume*, 39° 38' 41.85"N; 2° 39' 8.79"E). Livestock production was kept-differed between farms (Table 20). In *Can Cosme* ovine was the main production, whereas *Son Ajaume* showed ovine, pig and poultry production. *Can Cosme* had also chicken, a single horse outdoors and also a pigsty located indoors. The animals in *Son Ajaume* were all located outdoors and livestock was composed by more species including donkeys, different avian species such as doves, turkeys, ducks, gooses and helmed guineafowls (*N. meleagris*) The pig density was higher in *Son Ajaume* than in *Can Cosme*.

Collections were conducted using two UV light-suction traps per farm (Onderstepoort model; OVI-ARC, Onderstepoort, South Africa) during 24 non-consecutive nights (12 nights in each farm) from dusk to dawn between April 30th and November 17th 2015. No BTV was circulating during that period. Traps were separated 30 m from each other avoiding light interference between the traps.

Captures were collected early in the morning of the following day to avoid high mortality of adults, for this, trap collectors were filled with moistened paper to provide humid shelter for insects.

Figure 56. Location of the two sampled livestock farms in Majorca Island.

Table 20. Species and number of animals in the sampled farms *Can Cosme* and *Son Ajaume*.

Species	***Can Cosme***	***Son Ajaume***
Bos taurus	26	27
Ovis aries	535	147
Capra hircus	7	55
Equus caballus	1	2
Equus asinus	-	4
Equus caballus x asinus	-	1
Sus scrofa domestica	16	206
Gallus gallus	25	50
Numida meleagris	-	30
Meleagris gallopavo	-	25
Anser anser	-	5
Anas platyrhynchos domesticus	-	40
Columba palumbus	15	150
Total	625	742

After collection, active *Culicoides* midges were separated into a gauze cage from the non-targeted insects and anesthetized with cold following the procedures of Venter *et al*. (1991). Gravid females were selected (Dyce 1969) and kept individually in 64x50 mm cardboard boxes (Watkins & Doncaster®). Boxes were provided with 5 cm plastic Petri dish at the bottom with moistened cotton wool and filter paper as substrate for oviposition (Fig. 34). Paper pads of 10% sucrose solution were used for female's feeding, mixed with 0.2% of Nipagin (Methyl *p*-hydroxybenzoate) as an antibiotic to prevent fungal proliferation (Veronesi *et al*. 2009). After oviposition, eggs were counted and transferred to 100 mm Petri dishes filled with 10 ml of 2% European Bacteriological Agar gel medium and humidified daily to prevent desiccation (Boorman 1985). Adults and eggs/larvae were all kept separated inside climatic chambers (Climas® Grow chamber) at three different temperatures and humidity: 18.5 ± 0.9°C with 56.3 ± 4.1 % relative humidity (RH) (abbreviated as 18ºC), 24.3 ± 0.5°C with 63.9 ± 2.2 % RH (abbreviated as 25ºC) and 30.4 ± 0.5°C and 82.6 ± 4.5 % RH (abbreviated as 30ºC).

For larvae feeding, we provided *Panagrellus redivivus* species (Nematoda: Panagrolaimidae) grown in cereal powder (Kettle *et al*. 1975, Boorman 1985). Larvae were daily measured using a Stereomicroscope (Nikon® SMZ 645) to measure the larval growth according to rank size of the total length of larvae estimated by several authors (Kettle and Lawson 1952, Becker 1960, Murphree and Mullen 1991, Slama *et al*. 2013). Pupae were counted and transferred from the agar medium again to the cardboard boxes where F1 adults emerged and were fed daily with 10% sucrose solution.

Culicoides females were identified using keys based on its wing pattern (Rawlings 1996, Mathieu *et al*. 2012). Due to the difficulty in discriminating between females of the Obsoletus complex species, males from the F1 were identified according to the keys of Campbell and Pelham-Clinton (1960), Delécolle (1985) and Nielsen and Kristensen (2011).

The Shannon's diversity index (*H'*) (Shannon and Weaver 1949) was calculated in order to measure the diversity of total *Culicoides* females from both farms. Results were analysed using the SPSS Statistics® 17.0 software. Analyses were subjected to non-parametric Kolmogórov-Smirnov two-sample test (K-S) for assessing the differences of bionomic parameters between temperatures.

7.3. Results

7.3.1. Species composition and survival in sampling

A total of 7657 *Culicoides*, included in six different subgenera, were collected from both farms: 4862 individuals being 16.5 % gravid females from *Can Cosme* and 2795 from *Son Ajaume* being 48.9 % gravid females. Total survival rates in light traps were 34.5% ($\bar{x}$= 30.7 ± 4.2%) in *Can Cosme* and 23.3% ($\bar{x}$= 26.1 ± 9.5%) in *Son Ajaume*. Abundance of the Obsoletus complex was higher in *Can Cosme* while that of *C. imicola* was higher in *Son Ajaume*. In fact, not only the number of total and gravid females differed within farms but also the species composition. *Son Ajaume* exhibited higher biodiversity than *Can Cosme* (*H'*= 2.265, table 21) showing two species that not appeared in *Can Cosme*: *Culicoides parroti* Kieffer and *Culicoides brunnicans* Edwards.

Table 21. Number of alive gravid and total *Culicoides* species collected in both livestock farms with sampling survival rates. In brackets: total individuals. GF: gravid females.

	Can Cosme			***Son Ajaume***		
Species	**GF**	**Alive**	**Survival %**	**GF**	**Alive**	**Survival %**
Subgenus *Avaritia*						
Obsoletus complex	106 (257)	1230 (3432)	35.8%	6 (33)	43 (179)	24.0%
C. imicola	3 (14)	11 (48)	22.9%	45 (162)	177 (954)	18.6%
Subgenus *Beltranmyia*						
C. circumscriptus	108 (296)	219 (684)	32.0%	218 (750)	274 (992)	27.6%
Subgenus *Culicoides*						
C. newsteadi	13 (30)	76 (229)	33.2%	7 (20)	52 (163)	31.9%
C. puncticollis	1 (6)	3 (13)	23.1%	3 (6)	5 (23)	21.7%
Subgenus *Monoculicoides*						
C. parroti	0	0	-	2 (4)	6 (8)	75.0%
Subgenus *Oecacta*						
C. brunnicans	0	0	-	1 (1)	1 (1)	100%
C. comosioculatus Tokunaga	0	0 (1)	0%	4 (6)	4 (6)	66.7%
Subgenus *Sensiculicoides*						
C. cataneii	40 (146)	60 (204)	29.4%	47 (230)	49 (251)	19.5%
C. jumineri	3 (11)	4 (12)	33.2%	1 (1)	3 (3)	100%
Incertae sedis						
C. paolae	10 (41)	73 (239)	30.5%	25 (153)	37 (215)	17.2%
Total	284 (801)	1676 (4862)	34.5%	359 (1366)	651 (2795)	23.3%
		H’= 1.475			*H’*= 2.265	

H’: Shannon’s diversity index

7.3.2. Oviposition

The total percentage of females that oviposited among all species was 15.7% at 18ºC, 10.4% at 25ºC and 11.6% at 30ºC. Only 4 species managed to oviposit. The Obsoletus complex species and *C. circumscriptus* laid eggs at the three different temperatures. The Obsoletus complex showed the highest percentage of oviposition at 18ºC (66.7% in *Son Ajaume*) (Fig. 57 B). The species *C. circumscriptus* exhibited higher percentages of oviposition at 18ºC and 30ºC (27.3% in *Son Ajaume*). *Culicoides paolae* only oviposited at 18ºC in *Can Cosme* and at 30ºC in *Son Ajaume,* whereas *C. cataneii* only oviposited in *Can Cosme* at 18ºC. The other collected species did not lay eggs at any temperature.

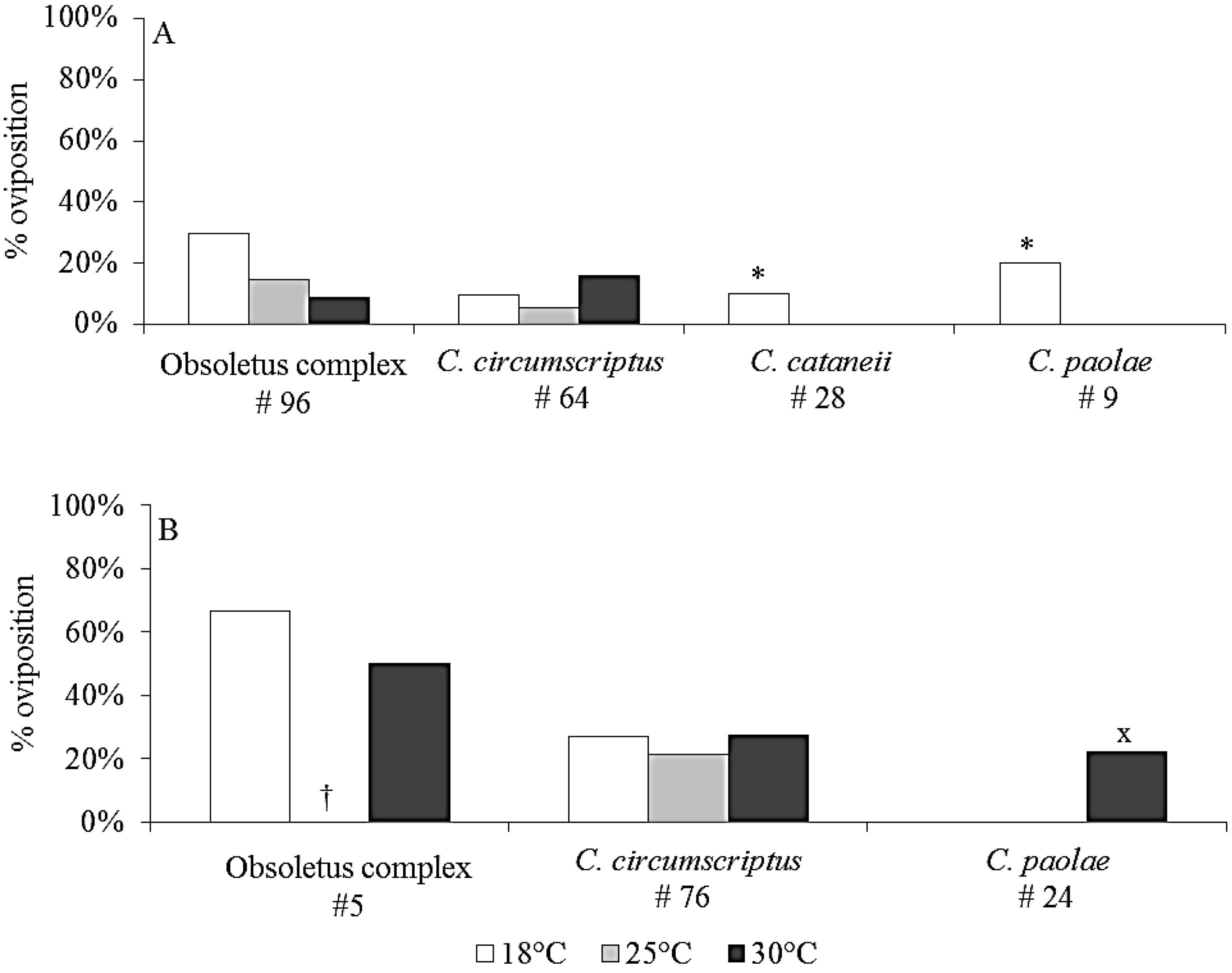


Figure 57. Percentage of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) that oviposited at different temperatures. (#): Sample size. (*): The species only oviposited at 18ºC. (x): The species only oviposited at 30ºC. (†): The individuals at 25º did not survive.

The average time to oviposit among all species was 20.0 ± 17.2 days at 18ºC, 12.5 ± 9.7 days at 25ºC and 5.5 ± 4.8 at 30ºC (Table 22). These results showed that time to oviposit were longer at lower temperatures (Fig. 58). Values recorded from *C. circumscriptus* at 18ºC in *Son Ajaume* exhibited significant longer time to oviposit than the individuals kept at 30ºC (2-tailed K-S test, *P*<0.05). In fact, one individual of *C. circumscriptus* showed the highest time to oviposit (59 days) at 18ºC. Obsoletus complex showed shorter times to oviposit at any temperature either at *Can Cosme* or *Son Ajaume* with average values lower than 15 (Table 22 and Fig. 58).

Table 22. Time to oviposit of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. In brackets: Sample size. (c): Significant differences with respect to 30ºC (K-S, $P<0.05$).

Species	**F**	**18ºC**		**25ºC**		**30ºC**	
		$\bar{x}$ days ± S.D.	**Max/Min. days**	**$\bar{x}$ days ± S.D.**	**Max/Min. days**	**$\bar{x}$ days ± S.D.**	**Max/Min. days**
Obsoletus complex	**A**	14.3 ± 17.0 (8)	54/1	8.1 ± 6.9 (5)	20/3	2.3 ± 1.2 (3)	3/1
	B	2 (2)	2/2	-	-	3 (1)	3/3
C. circumscriptus	**A**	44.0 ± 21.2 (2)	59/29	1 (1)	1/1	4.4 ± 5.1 (4)	12/1
	B	25.1^{c} ± 13.9 (7)	48/10	18.1 ± 9.3 (6)	30/8	7.8 ± 5.1 (6)	17/2
C. cataneii	**A**	22 (1)	22/22	-	-	-	-
	B	-	-	-	-	-	-
C. paolae	**A**	17 (1)	17/17	-	-	-	-
	B	-	-	-	-	7.0 ± 7.1 (2)	12/2
Total average	**A**	20.0 ± 18.9 (12)	59/1	6.9 ± 6.8 (6)	20/1	3.5 ± 3.8 (7)	12/1
	B	19.9 ± 15.7 (9)	48/2	18.1 ± 9.3 (6)	30/8	7.1 ± 5.0 (9)	17/2
	T	20.0 ± 17.2 (21)	59/1	12.5 ± 9.7 (12)	30/1	5.5 ± 4.8 (16)	17/1

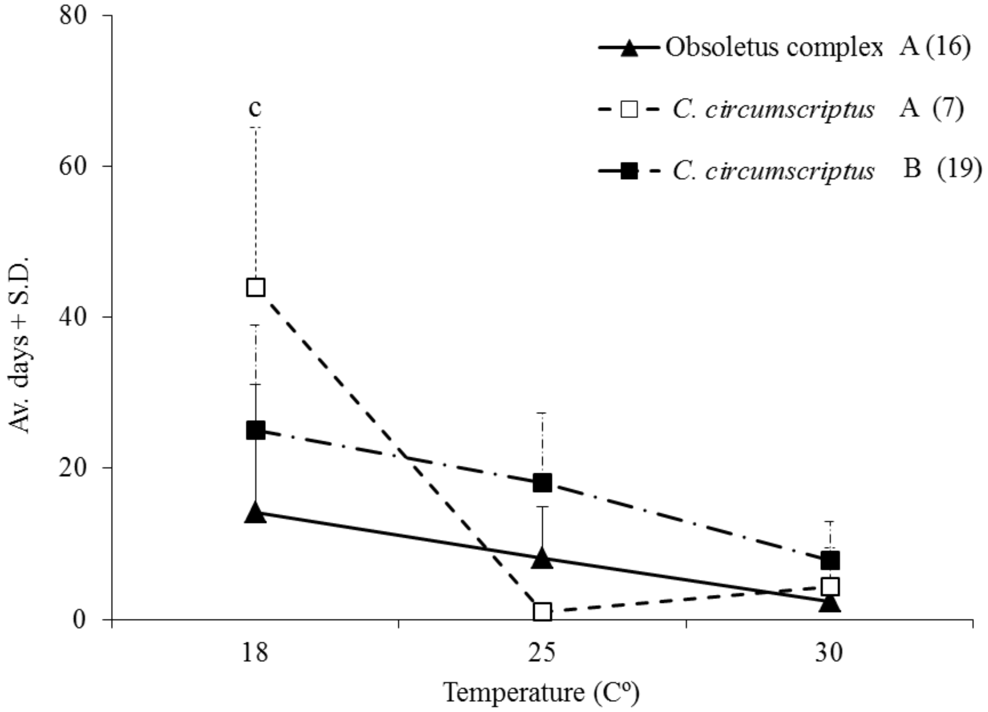


Figure 58. Average time to oviposit (in days) ± S. D. of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. Only species with data from the three temperatures are represented. (c): Significant differences with respect to the 30ºC (K-S, *P*<0.05). In brackets: Sample size.

The average number of eggs laid among all *Culicoides* gravid females was 62.9 ± 38.4 eggs / female at 18ºC; 82.9 ± 55.0 eggs / female at 25ºC and 69.5 ± 49.9 eggs / female at 30ºC. The species *C. circumscriptus* showed the highest number of eggs: 210 ($\bar{x}$= 128.8 ± 81.1) from individuals collected in *Can Cosme* at 30ºC and 203 ($\bar{x}$= 109.5 ± 64.0) from individuals collected in *Son Ajaume* at 25ºC (Fig. 59).

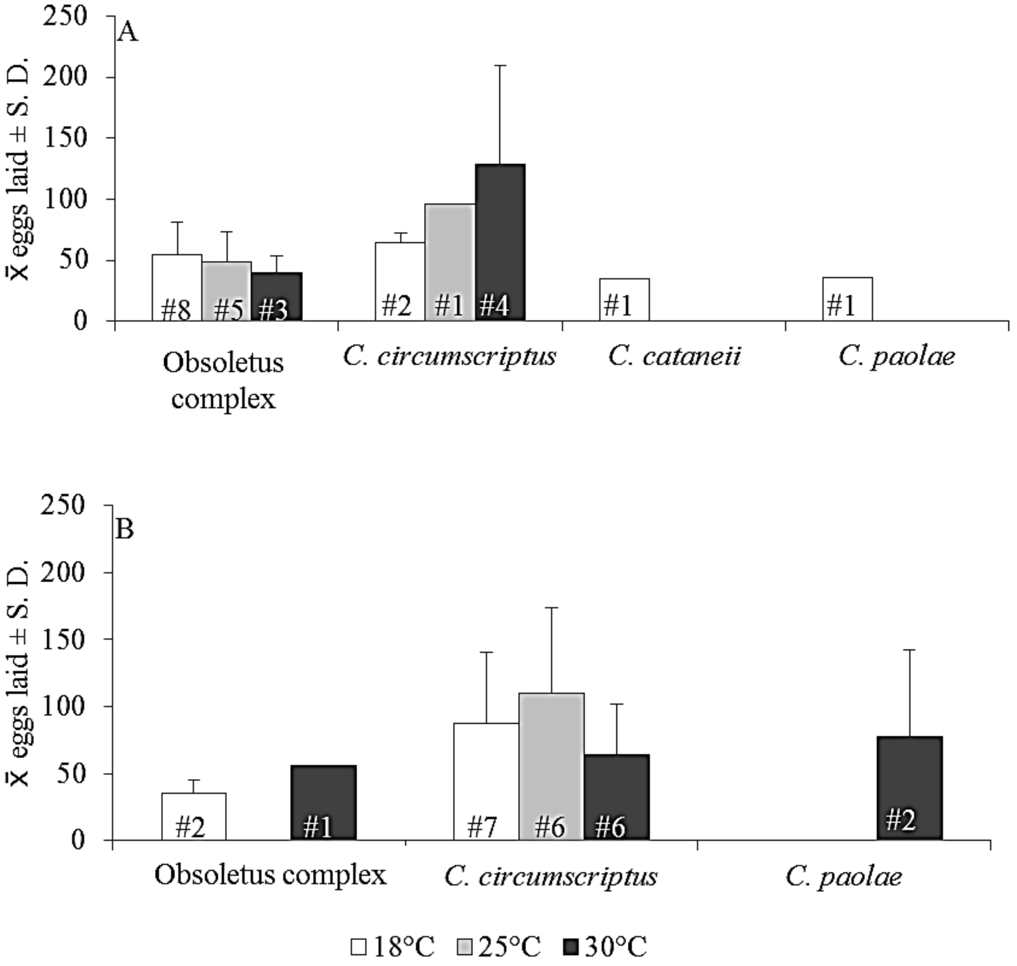


Figure 59. Average number of eggs laid ± S. D. of the field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. (#): Sample size.

7.3.3. Survival of field-collected gravid females

The average total lifespan of field gravid females was 23.4 ± 19.8 days for the individuals kept at 18ºC, 16.8 ± 13.7 days at 25ºC and 13.1 ± 9.2 days at 30ºC (Table 23). *Culicoides* species lived longer kept at 18ºC. In fact, the highest longevity recorded was 80 days for one individual of Obsoletus complex from *Can Cosme* as well as one individual of *C. circumscriptus* from *Son Ajaume* (Table 23 and Fig. 60). In addition, Obsoletus complex, *C. circumscriptus* and *C. paolae* showed significant longer lifespan at 18ºC than the other temperatures (2-tailed K-S test, $P<0.05$). *Culicoides imicola* showed similar lifespan at 18ºC and 25ºC both in *Can Cosme* and *Son Ajaume*. *Culicoides jumineri* from *Son Ajaume* lived longer at 30ºC and one individual of *C. cataneii* recorded the highest lifespan at 30ºC in *Can Cosme*, surviving 41 days. Regarding the lifespan after oviposition, *Culicoides* PF also showed longer lifespan at 18ºC (Table 24 and Fig. 61).

Table 23. Lifespan of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. In brackets: Sample size. (T): Total average. (a): Significant differences with respect to the other temperatures (2-tailed K-S test, *P*<0.05). (b): Significant differences with respect to 25ºC (2-tailed K-S test, *P*<0.05). (c): Significant differences with respect to 30ºC (2-tailed K-S test, *P*<0.05).

Species	**F**	**18ºC**		**25ºC**		**30ºC**	
		$\bar{x}$ days ± S.D.	**Max/Min. days**	**$\bar{x}$ days ± S.D.**	**Max/Min. days**	**$\bar{x}$ days ± S.D.**	**Max/Min. days**
Obsoletus complex	**A**	29.7[a] ± 24.6 (27)	80/2	14.5 ± 14.6 (34)	54/1	16.1 ± 9.8 (35)	40/1
	B	19.3 ± 15.0 (3)	35/5	-	-	10.5 ± 10.6 (2)	18/3
C. circumscriptus	**A**	22.9[b] ± 20.2 (21)	65/1	13.4 ± 11.6 (18)	36/1	14.2 ± 10.2 (25)	34/1
	B	24.8[c] ± 19.7 (26)	80/1	21.5 ± 14.3 (28)	52/1	11.7 ± 7.9 (22)	22/1
C. imicola	**A**	29 (1)	29/29	28 (1)	28/28	-	-
	B	21.5 ± 14.6 (13)	42/2	22.3 ± 11.5 (11)	46/5	8.6 ± 6.5	18/1
C. cataneii	**A**	23.4 ± 18.8 (10)	62/1	19.0 ± 18.4 (9)	46/1	16.7 ± 12.6 (10)	41/1
	B	14.4 ± 15.5 (8)	41/1	7.7 ± 9.8 (3)	19/2	13.6 ± 7.1 (8)	26/1
C. newsteadi	**A**	30.8 ± 27.6 (4)	65/3	15.5 ± 11.5 (4)	32/6	13.4 ± 9.3 (5)	23/3
	B	21.3 ± 17.2 (3)	35/2	-	-	11.0 ± 7.4 (4)	18/1
C. paolae	**A**	24.8[a] ± 17.7 (5)	49/8	-	-	2.5 ± 2.4 (4)	6/1
	B	11.7 ± 13.9 (9)	43/1	9.0 ± 5.4 (6)	17/14	11.7 ± 6.1 (9)	22/2
C. jumineri	**A**	3 (1)	3/3	-	-	11 (1)	11/11
	B	-	-	-	-	26 (1)	26/26

Table 23. (Continued)

Species		18ºC		25ºC		30ºC	
	F	x̄ days ± S.D.	Max/Min. days	x̄ days ± S.D.	Max/Min. days	x̄ days ± S.D.	Max/Min. days
C. puncticollis	**A**	-	-	-	-	-	-
	B	30 (1)	30/30	-	-	-	-
C. parroti	**A**	-	-	-	-	-	-
	B	22-0 ± 25.5 (2)	40/4	-	-	19 (1)	19/19
C. brunnicans	**A**	-	-	-	-	-	-
	B	-	-	17 (1)	17/17	-	-
Total average	**A**	26.0 ± 21.8 (69)	80/1	15.1 ± 14.0 (66)	54/1	14.1 ± 10.2 (79)	41/1
	B	20.5 ± 17.0 (64)	80/1	19.2 ± 13.2 (49)	52/1	11.6 ± 7.3 (56)	26/1
	T	23.4 ± 19.8 (133)	80/1	16.8 ± 13.7 (116)	54/1	13.1 ± 9.2 (135)	41/1

Table 24. Lifespan after oviposition of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. In brackets: Sample size. (T): Total average. (*): The species only oviposit at 18ºC. (#): The species only oviposit at 30ºC.

Species		**18ºC**			**25ºC**			**30ºC**		
	F	**$\bar{x}$ days ± S.D.**	**Max. days**	**Min. days**	**$\bar{x}$ days ± S.D.**	**Max. days**	**Min. days**	**$\bar{x}$ days ± S.D.**	**Max. days**	**Min. days**
Obsoletus comp.	**A**	23.9 ± 24.4 (8)	70	1	6.7 ± 4.3 (5)	13	1	12.7 ± 10.0 (3)	24	5
	B	18.0 ± 21.2 (2)	33	3	-	-	-	1 (1)	0	0
C. circumscriptus	**A**	19.5 ± 23.3 (2)	36	3	3 (1)	3	3	4.1 ± 1.2 (4)	5	2
	B	16.9 ± 10.4 (7)	32	5	6.9 ± 4.5 (6)	13	2	5.0 ± 5.6 (6)	11	1
C. cataneii	**A**	40* (1)	40	40	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-
C. paolae	**A**	32* (1)	32	32	-	-	-	-	-	-
	B	-	-	-	-	-	-	0.5$^{\#}$ ± 0.7 (2)	1	1
Total average	**A**	25.4 ± 21.2 (12)	70	1	6.1 ± 4.1 (6)	13	1	7.8 ± 7.4 (7)	24	2
	B	17.2 ± 11.9 (9)	33	3	6.9 ± 4.5 (6)	13	2	2.7 ± 4.3 (7)	11	1
	T	22.1 ± 18.1 (21)	70	1	6.5 ± 4.1 (12)	13	1	5.4 ± 6.5 (14)	24	1

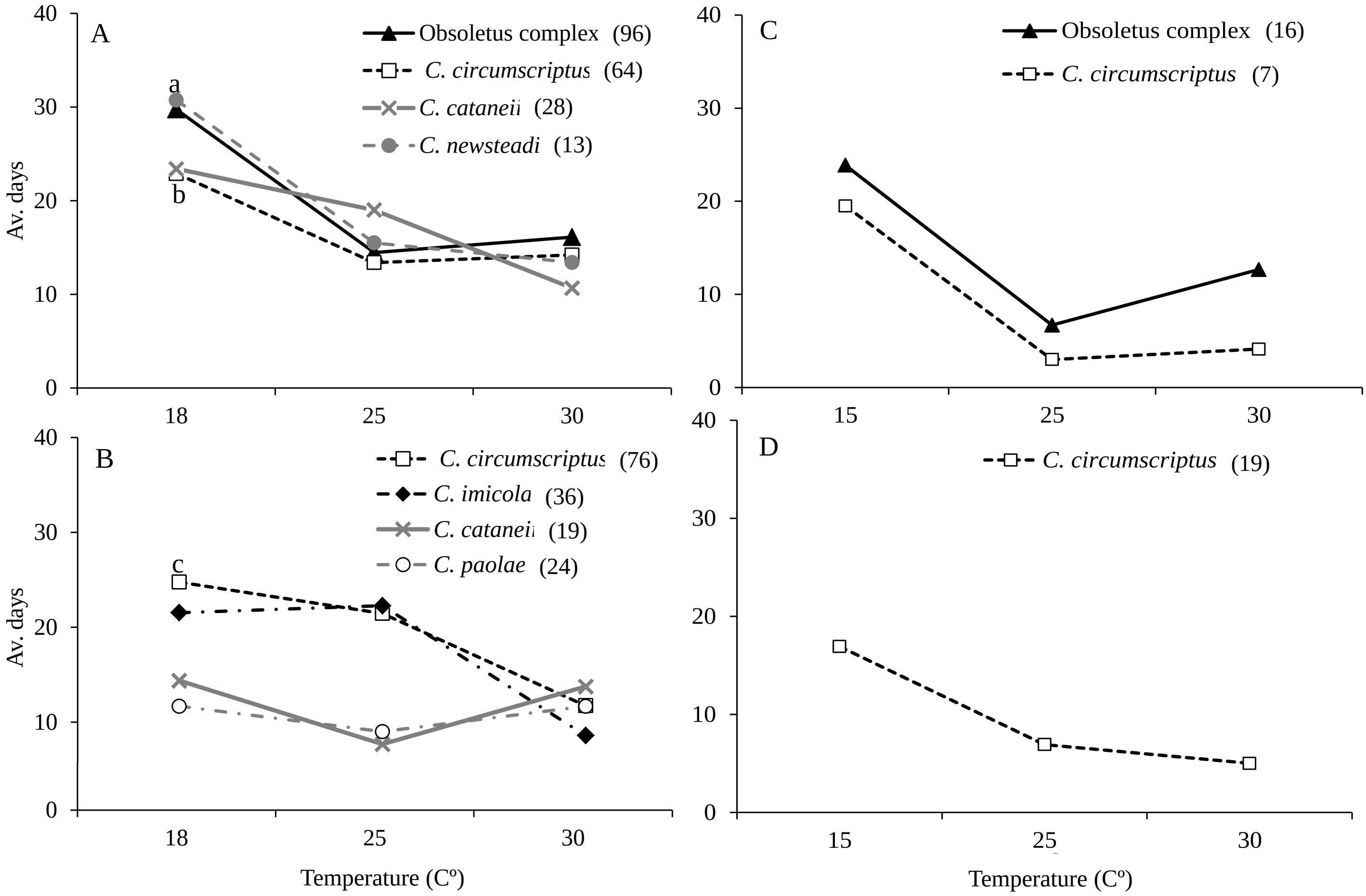


Figure 60. Lifespan of field gravid females from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures and the lifespan after oviposition in *Can Cosme* (C) and *Son Ajaume* (D). Only species with data from the three tested temperatures are represented. In brackets: Sample size. (a): Significant differences with respect to the other temperatures (2-tailed K-S test, *P*<0.05). (b): Significant differences with respect to 25ºC (2-tailed K-S test, *P*<0.05). (c): Significant differences with respect to 30ºC (2-tailed K-S test, *P*<0.05).

7.3.4. Life-cycle and F1 lifespan

The duration of each life-cycle stage increased as temperatures decreased. The longest life-cycle was 85.5 and 81.2 days for *C. circumscriptus* at 18ºC from *Can Cosme* and *Son Ajaume* respectively (Fig. 61). The longest life stage was the F1 adult's lifespan, reaching the maximum of 98 days in case of one individual of *C. circumscriptus* followed by 88 days for one individual of *C. obsoletus* at 18ºC from *Can Cosme*. Adults of *C. obsoletus* were unable to complete its life-cycle at 30ºC, whereas *C. paolae* only finished its life-cycle at that temperature. In fact, *C. paolae* recorded the shortest life-cycle period at 30ºC (32.6 days; Fig. 61 B). The eggs of *C. cataneii* did not hatch at 18ºC.

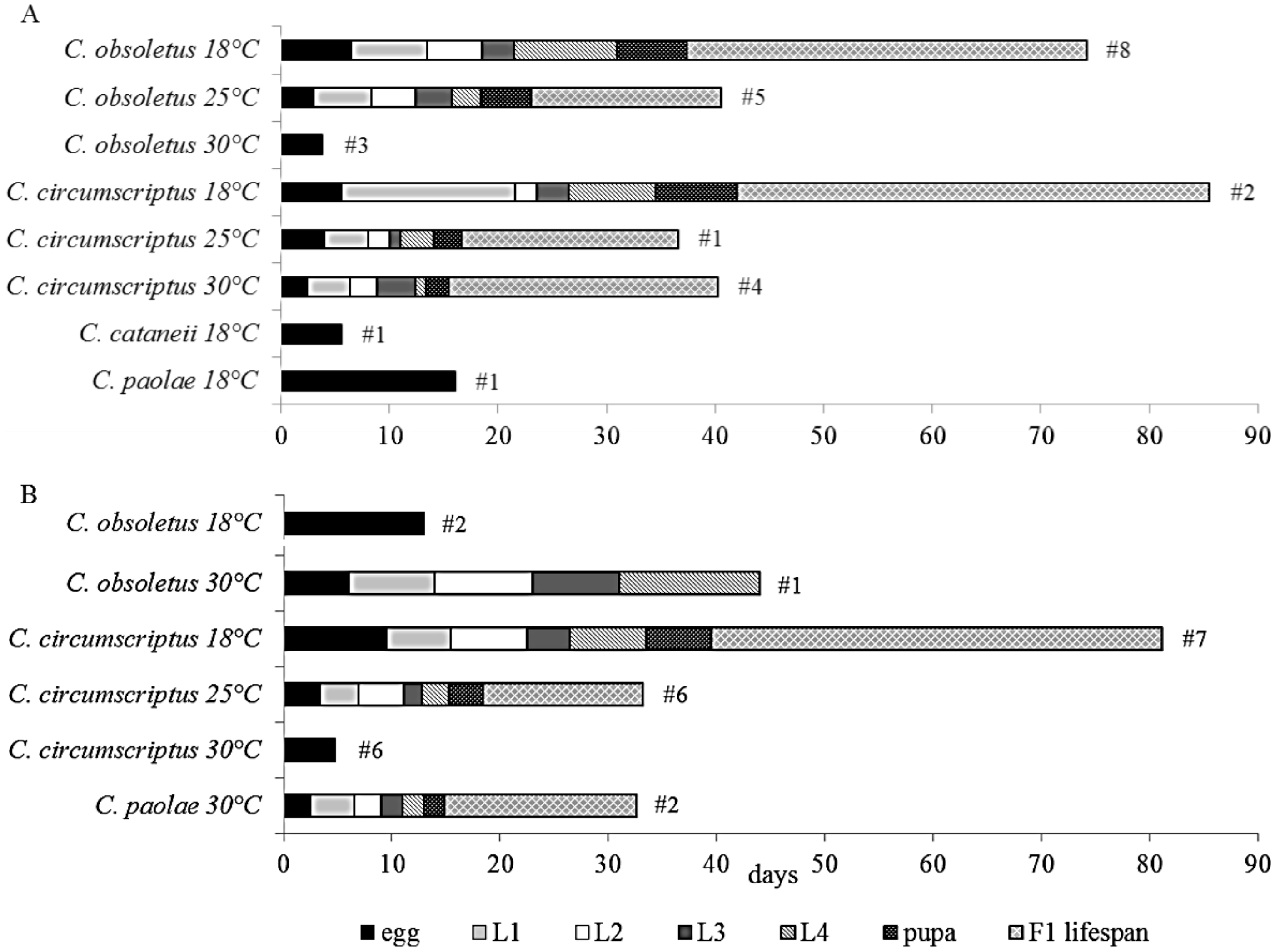


Figure 61. Average sub-adult stages duration in days and F1 adult survival of each *Culicoides* species from *Can Cosme* (A) and *Son Ajaume* (B). (#): Sample size.

7.3.5. Percentage of egg hatching, pupation success and adult emergence

The total average of egg hatching among all species was 26.7 ± 31.3% for the individuals kept at 18ºC, 31.8 ± 34.6% at 25ºC and 13.8 ± 20.4% at 30ºC. *Culicoides obsoletus* showed the highest percentage of egg hatching at 18ºC, while for *C. circumscriptus* and *C. paolae* was at 25ºC and 30ºC respectively (Fig. 62).

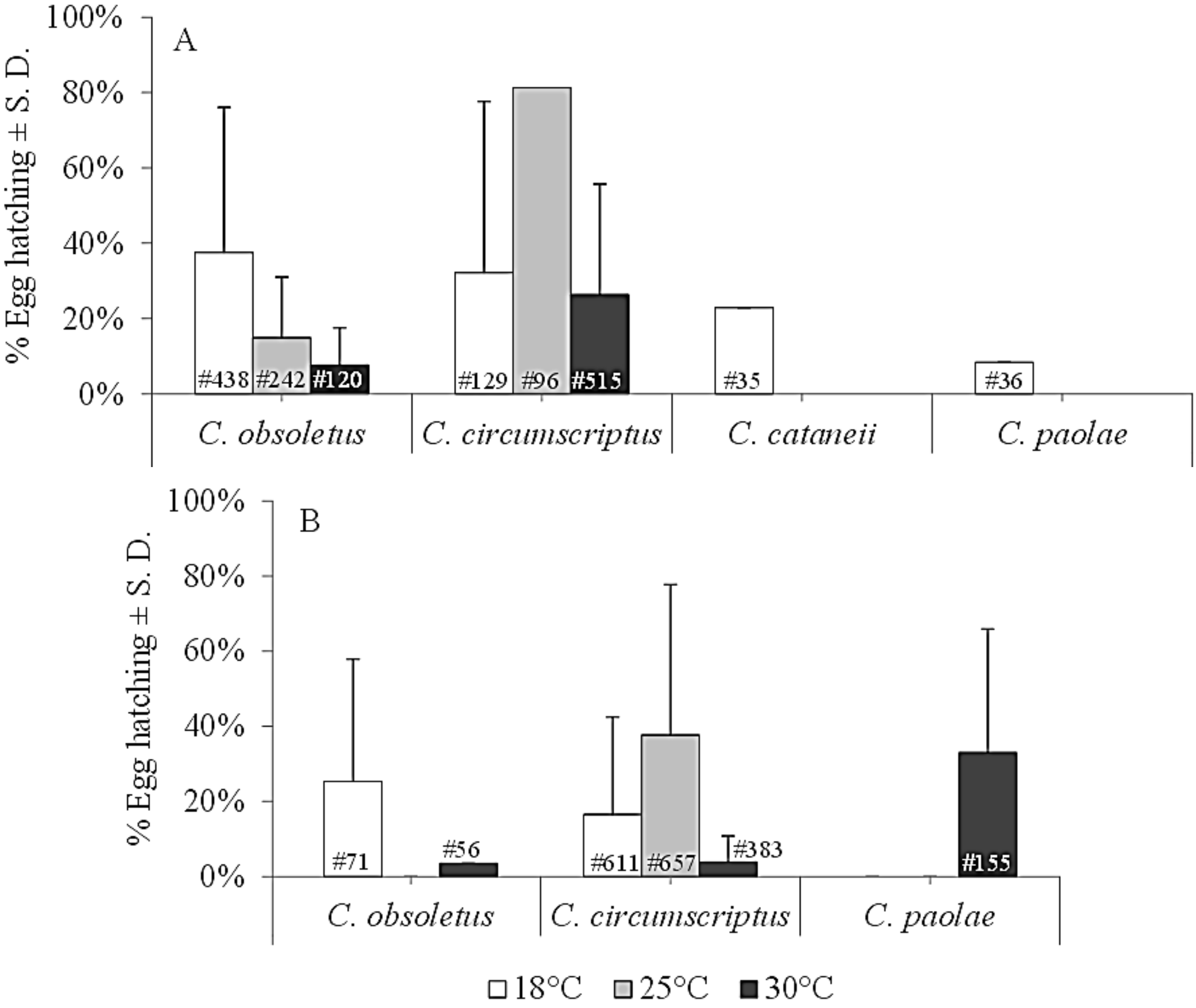


Figure 62. Average percentage ± S. D. of egg hatching obtained from *Culicoides* field gravid females in *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures. (#): Sample size.

Total averages of percentage of pupation were 13.9 ± 19% at 18ºC, 33.5 ± 30% at 25ºC and 25.3 ± 33.6% at 30ºC. The species *C. obsoletus* and *C. circumscriptus* showed higher percentages of pupation at 25ºC (Fig. 63) while *C. paolae* showed the highest percentage of pupation at 30ºC (65.3%). *Culicoides cataneii* and *C. paolae* from *Can Cosme* and *C. obsoletu*s from *Son Ajaume* died at larvae stage.

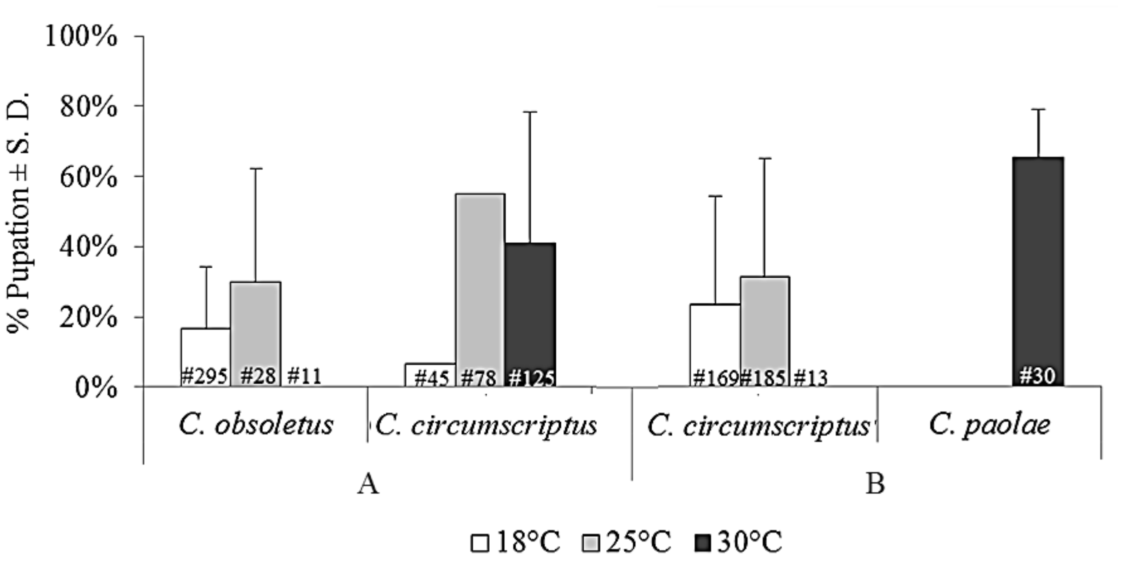


Figure 63. Average percentage ± S. D. of pupation of F1 *Culicoides* species from *Can Cosme* (A) and *Son Ajaume* (B). (#): Sample size.

The percentage of adult emergence showed high values in general and was higher at high temperatures; the total average among all species was 58.3 ± 47.4% at 18ºC, 86.8 ± 26.4% at 25ºC and 95.5 ± 11.0% at 30ºC. In case of *C. paolae*, this percentage reached the 100% at 30ºC in *Son Ajaume* (Fig. 64). F1 adults of *C. circumscriptus* emerged at any temperature. *Culicoides obsoletus* at 25ºC in *Can Cosme* showed significantly higher percentage of adult emergence than the ones kept at 30ºC (2-tailed K-S test, *P*<0.05).

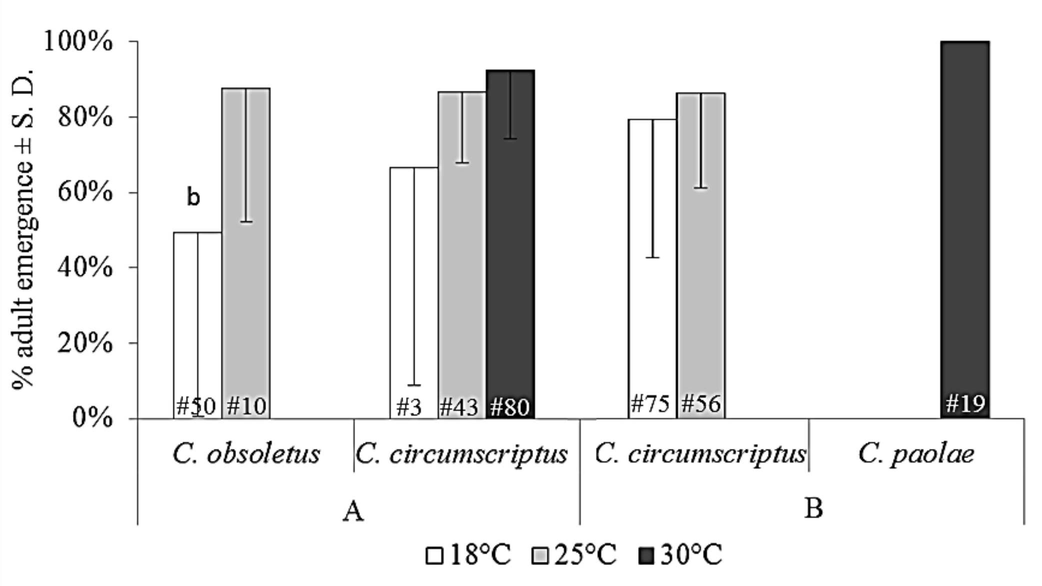


Figure 64. Average percentage ± S. D. of adult emergence of F1 *Culicoides* species from *Can Cosme* (A) and *Son Ajaume* (B). (b): Significant differences between both temperatures (2-tailed K-S test, *P*<0.05). (#): Sample size.

7.3.6. Pupation

Pupation of *C. obsoletus* occurred between the 5th and the 80th day (a period of 75 days) at 18ºC (Fig. 65), whereas L4 of this species finished pupating earlier (less than 50 days) at 25ºC. However, number of larvae that pupate at 25ºC where lower than the ones at 18ºC since the highest number of pupae recorded at 25ºC were 3 on the 4th day.

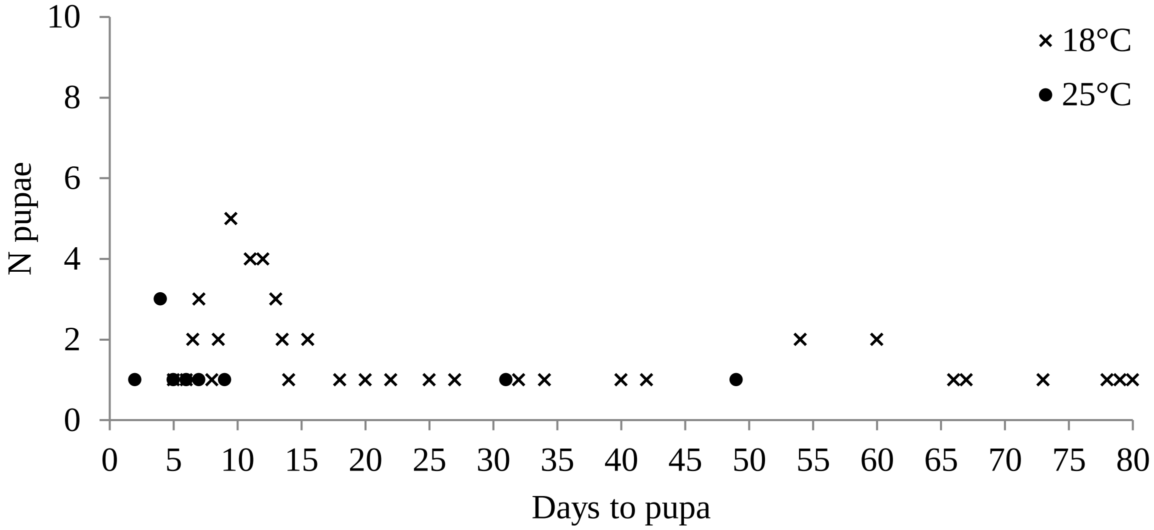


Figure 65. Number of daily L4 larvae of *C. obsoletus* that pupated from *Can Cosme* at 18ºC and 25ºC.

The L4 larvae of *C. circumscriptus* started to pupate earlier than *C. obsoletus* and were generally concentrated during the first 10 days (Fig. 66). Moreover, number of daily pupae of this species was higher than *C. obsoletus* and increase proportionally with the temperature, reaching values of 30 pupae during the 3rd day at 30ºC from individuals collected in *Can Cosme*. Although the pupation period of *C. circumscriptus* from *Son Ajaume* was shorter at 25ºC, number of pupae recorded per day of this species was higher and more evenly distributed at 18ºC.

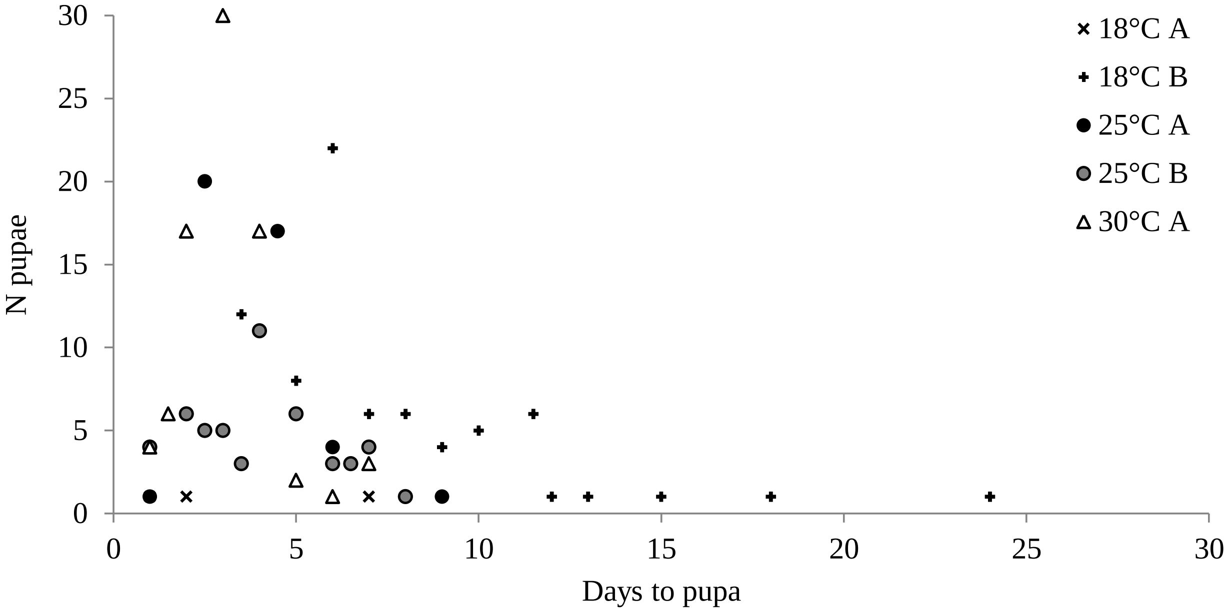


Figure 66. Number of daily L4 larvae of *C. circumscriptus* that pupated from *Can Cosme* (A) and *Son Ajaume* (B) at 18ºC, 25ºC and 30ºC.

Culicoides paolae larvae from *Son Ajaume* showed a similar pupation period than *C. circumscriptus* at 25ºC. Pupation of this species finished the 6th day after the first L4 larvae. The day of maximum number of pupae was 8 during the 2nd day of the pupation period (Fig. 67).

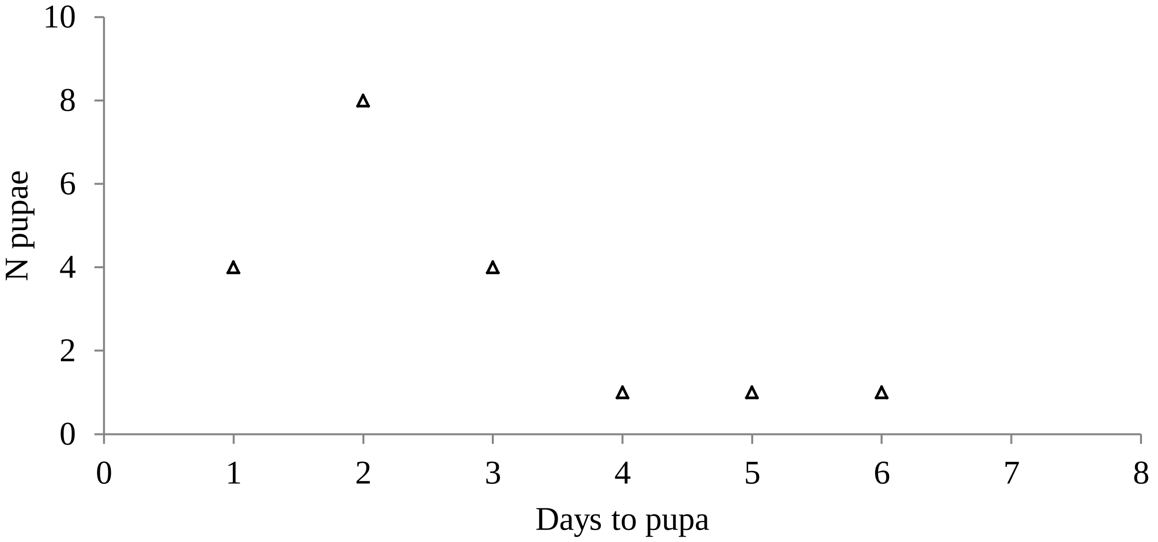


Figure 67. Number of daily L4 larvae of *C. paolae* that pupated from *Son Ajaume* at 30ºC.

7.3.7. Larvae growing

Larvae reared at 25ºC showed higher growing rates than the other temperatures (Fig. 68). The maximum values recorded were for *C. circumscriptus* at 25ºC, reaching 0.6 ± 0.1 mm per day in *Can Cosme* and 0.5 ± 0.2 mm/day in *Son Ajaume*. The larvae of *C. obsoletus* and *C. circumscriptus* growing at 18ºC exhibited significantly lower growth rates than at the other temperatures (2-tailed K-S test *P*<0.05). *Culicoides paolae* showed similar growth rates to *C. circumscriptus* at 30ºC.

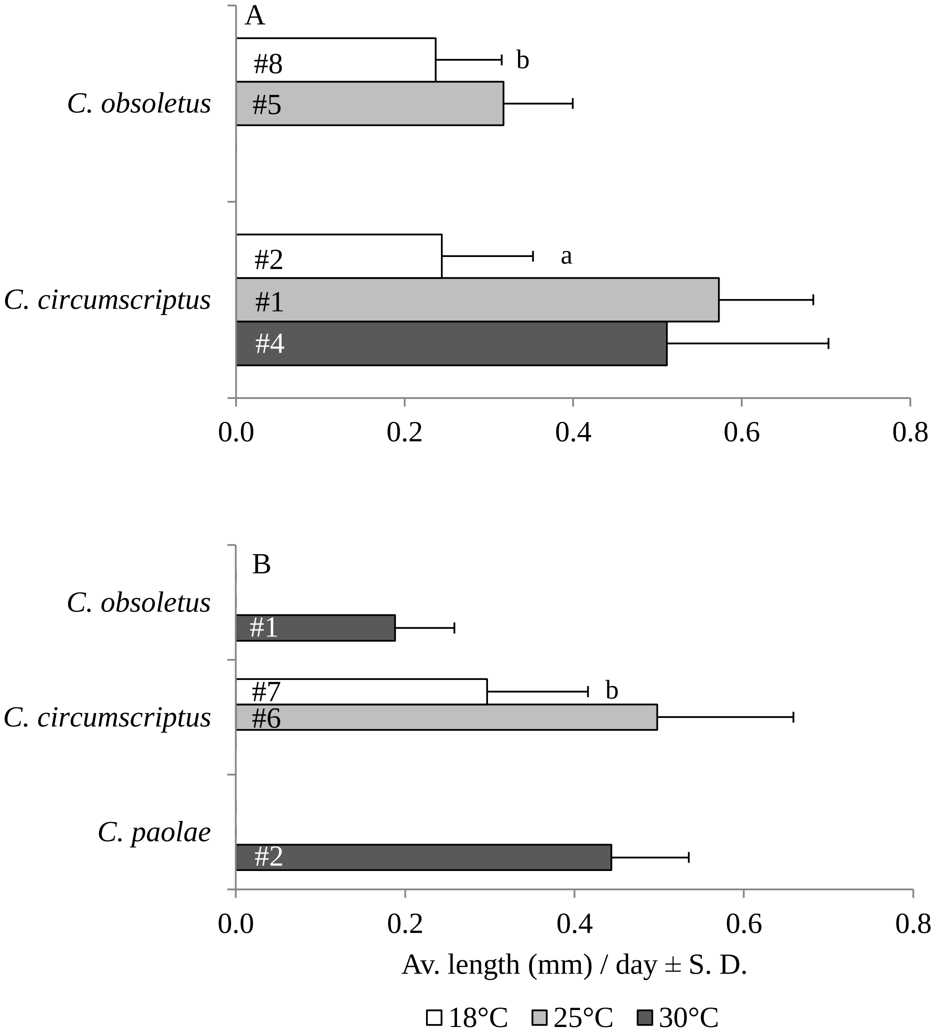


Figure 68. Average length of larval growth (in mm per day) ± S. D. of each species from *Can Cosme* (A) and *Son Ajaume* (B) at different temperatures in laboratory conditions. (a): Significant differences of 18ºC with respect the other temperatures. (b): Significant differences between 18ºC and 25ºC (2-tailed K-S test *P*<0.05). (#): Sample size.

7.3.8. Sex ratio

The species *C. circumscriptus* and *C. paolae* showed similar ratio of males to females between the three temperatures; with the exception of *C. circumscriptus* from *Can Cosme*, which had a 100% female progeny at 18ºC (Fig. 69). *Culicoides obsoletus* were male biased reaching a 100% of males from pupae kept at 18ºC from *Can Cosme*.

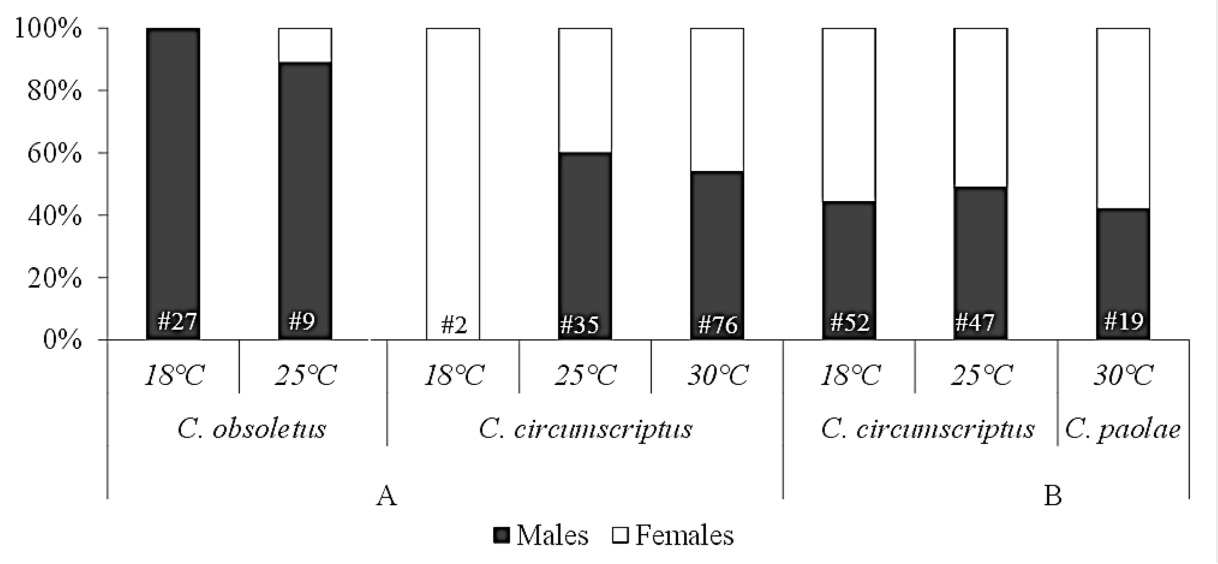


Figure 69. Percentage of total adults males and females for each species that emerged at different temperatures) in the laboratory from *Can Cosme* (A) and *Son Ajaume* (B). (#): Sample size

7.4. Discussion

7.4.1. Species composition and survival in sampling

The survival rates in both farms during the sampling with UV light traps, especially in *Son Ajaume*, did not exceed the 30%. This results were similar than values recorded by Del Río *et al*. (2014a) where species such as *C. imicola* also showed survival rates lower than 30% using the same sampling method. The moistened paper set in the traps collector was insufficient to maintain the *Culicoides* alive until its transport to the laboratory. The decrease of temperature during the night and the fan drying effect were probably unfavourable for *Culicoides* individuals. Sampling improvements such as a decrease of fan power or special chambers avoiding the direct effect of the fan (e.g. chambers with shelters for the midges) should be considered.

Culicoides species composition and the survival rates after collection with UV light traps were different between the two sampling sites. *H’* value determines the diversity in ecosystems. Values up to 2 such as in *Son Ajaume* are considered to be highly diverse (Shannon and Weaver 1949). Differences between sites in species composition could be linked to the hosts available in the farms or even the position of the traps. *Culicoides* abundance can vary by several orders of magnitude over short distances within a few hundred metres being; so, blood feeding preferences (Ninio *et al*. 2011, Martínez-de la Puente *et al*. 2012, 2015, Viennet *et al*. 2012), different substrate composition of the breeding sites (Foxi and Delrio 2010, Harrup *et al*. 2013) or also the position of the traps in relation to physical structures (Venter *et al*. 2018), could determine the observed differences in *Culicoides* diversity and abundance between farms. *Culicoides parroti* and *C. brunnicans* were only collected in *Son Ajaume*. Prior studies demonstrated that *C. parroti* showed preferences to feed on pig (Martínez-de la Puente *et al*. 2015) which could be related with the high density of pig located outdoors in *Son Ajaume*. Conversely, the exclusive presence of *C. brunnicans* in *Son Ajaume* was unexpected since this species feed on cattle and sheep (Garros *et al*. 2011, Ninio *et al*. 2011, Calvo *et al*. 2012, Martínez-de la Puente *et al*. 2015), being less abundant in *Son Ajaume*. The phenomenon of high abundance of gravid females, especially of *C. circumscriptus*, collected in both farms and also in the previous chapter remains unknown, but was probably related with long gonotrophic cycles of this species (Carpenter *et al*. 2006b).

7.4.2. Oviposition

As well as the previous chapter, the total percentage of oviposition on artificial substrate at different temperatures (18ºC, 25ºC and 30ºC) recorded in this study indicates that the substrate and the technique were adequate to obtain eggs from field-collected females. However, less than 20% of total gravid females collected in both farms managed to lay eggs (Obsoletus complex and the ornithophilic species *C. circumscriptus*, *C. paolae* and *C. cataneii*) and only two of them (Obsoletus complex and *C. circumscriptus*) oviposited eggs at the three temperatures. The vector species *C. imicola* and *C. newsteadi,* as well as the non-vectors *C. jumineri*, *C. puncticollis*, *C. parroti*, *C. brunnicans* and *C. comosioculatus* were less abundant in samples and did not succeed in ovipositing; however, the previous chapter demonstrated that, although rearing difficulties, oviposition of *C. imicola* and *C. newsteadi* can be successful under laboratory conditions. These low oviposition rates could be linked with the eggs absorption of some *Culicoides* females, related with the unsuitability of the artificial substrate (Carpenter *et al*. 2001). It previously mentioned that insects in field conditions are subjected to oscillations of temperature and the accumulation of that temperature may affect the reproduction metabolism of *Culicoides* females (Lühken *et al*. 2015). It should be considered that this is the first study using different temperatures for *C. newsteadi*, *C. paolae*, *C. cataneii* and *C. jumineri* and poor information is available about the bioecology of these species; thus, highlighting the novelty of the current study.

Biting midges kept at 18ºC showed higher oviposition rates than the ones kept at 25ºC and 30ºC. In fact, Obsoletus complex showed then highest oviposition rates at this temperature, being higher than the value of 7% recorded by Jamnback (1961) at 21.1 ºC. These results were probably related to its aforementioned capacity to adapt to different substrates.

The time taken for females to oviposit increased at the relative low temperatures used in this trial (18ºC). Results obtained for *C. obsoletus* were similar than that of Parker (1950), where oviposition took place between the first and the second week at 17.2ºC. Conversely, individuals kept at relative high temperatures (30ºC) oviposited earlier than other temperatures. These results agrees with a prior study of the life-cycle of *C. imicola* (Veronesi *et al*. 2009), demonstrating that these insects are highly temperature dependent.

The average number of eggs laid among all taxa demonstrated that fecundity of females was also variable between temperatures. Individuals kept at 30ºC laid more eggs than at other temperatures, except the Obsoletus complex from *Can Cosme* which laid more eggs at 18ºC. *Culicoides circumscriptus* laid the highest number of eggs at any temperature, possibly linked to the autogenous nature of this species (Kettle 1977) and its large body size (2.2 mm; Delécolle, 1985). However, the average eggs laid by this species were lower than other studies which obtained more than 250 eggs of *C. circumscriptus* from field gravid females (Becker 1960). The number of oviposited eggs by *C. obsoletus* at 18ºC and 25ºC were similar to previous studies (Hill 1947, Parker 1950, Braverman and Linley 1994) where *C. obsoletus* laid between 30 and 57 eggs at temperatures ranging the 17.2ºC and 21.1ºC. Conversely, Jamnback (1961) reported lower average number of eggs per female (14.7) at 21.1ºC than our results and the above mentioned works. The eggs absorption commented in the prior chapter or also bionomic particularities related to local populations and conditions on the artificial rearing could be considered as possible interpretations. In addition, fecundity may be affected by using artificial blood feeding or frozen blood (Hill 1947, Jamnback 1961) instead of natural or fresh blood feeding (Fahrner and Barthelmess 1988, Braverman and Linley 1994).

Culicoides cataneii, which only oviposited at 18ºC, recorded lower number of eggs than the value of 157.9 ± 7.4 obtained in that of Braverman and Linley (1994) at similar temperature. This difference may be related with the methodology used for obtaining eggs, since the authors of Braverman and Linley (1994) dissected field gravid females to obtain the eggs. This methodology allows to get all eggs from the abdomen avoiding the above commented eggs absorption from *C. cataneii* females.

The remaining species *C. paolae* only oviposited at 18ºC and 30ºC. This species showed the highest number of eggs laid at 30ºC in *Son Ajaume*, similar to the number of eggs laid by the largest species *C. circumscriptus* at this temperature. Conversely, the individuals kept at 18ºC showed similar number of eggs to *C. obsoletus* at that temperature. This was an unexpected result and may be related to the unknown fecundity aspects of this ornithophilic species.

7.4.3. Survival of field-collected gravid females

In the current study, individuals kept at 18ºC showed the highest lifespan, 23.4 ± 19.8 days in average. Adult *Culicoides* midges can survive between 10–20 days (Mellor *et al*. 2000) and some studies have demonstrated that lifespan of species such as *C. sonorensis* may extend during mild winter months where some adults can survive up to three months at 10°C (Lysyk and Danyk 2007, Purse *et al*. 2015). Therefore, survival has often been shown to increase when temperature decrease (Gerry and Mullens 2000, Mullens *et al*. 2004) and this is consistent with the observations in this study (except a single individual of *C. jumineri*). However, we used field collected gravid females with undetermined age and number of gonotrophic cycles, whereas the aforementioned studies used mainly NF and expected to live longer.

In Europe members, Obsoletus complex can be active at temperatures as low as 10°C (Versteirt *et al*. 2017) and, in laboratory conditions, *C. obsoletus* can survive more than 90 days at 17°C and up to 10 days at 4°C (Goffredo *et al*. 2004). In South Africa, the adults of *C. imicola* can survive for more than 15 days at 22.2ºC (Nevill 1971). These results are similar to the maximum of 80 days of lifespan recorded for *C. obsoletus* in our study at 18°C and the 21.5 and 29 days for *C. imicola* at 18ºC and 25ºC. The minimum temperature required for viral replication inside vectors is generally consistent for different arboviruses transmitted by *Culicoides* vector species (Carpenter *et al*. 2011). Previous studies have also demonstrated the capability of species from the Imicola group (*C. imicola* and *C. bolitinos*) to harbour BTV for more than 20 days at 10 °C (Paweska *et al*. 2002). However, *C. bolitinos*, which is a high altitude species, seems to be adapted to cooler temperatures than *C. imicola* and able to survive the relatively mild winter temperatures as adults in South Africa (Verhoef *et al*. 2014).

The maximum lifespan of *C. circumscriptus* at 18ºC also reached the 80 days and the average (22.9 ± 20.2) was higher than that of Becker (1960), where adults survive over two weeks at temperatures between 17°C and 20°C. These results could be related to the variations of temperature in the study of Becker (1960) versus the fixed temperature of 18ºC in the current study.

Adult lifespan was shorter in individuals kept at 30ºC. Not only temperatures affect adult survival and activity, but also other variables such as humidity, wind, and rainfall (Purse *et al*. 2015, Carpenter *et al*. 2008b). *Culicoides* individuals at 30ºC in the current study were kept at 82.6 ± 4.5 % RH. This high percentage of humidity could explain the low ratio of *Culicoides* survival. At high temperatures, high humidity (up to 85%) hinder the ability of midges to eliminate excess metabolic water by evaporation and was considered detrimental for species such as *C. sonorensis* (Wittmann *et al*. 2002). High humidity could be also related with fungi or other pathogens that can thrive in moist conditions and cause an increase of *Culicoides* mortality.

7.4.4. Life-cycle and F1 adult lifespan

Our results showed that the development of *Culicoides* larvae was delayed at low temperatures. The study of Lühken *et al*. (2015) demonstrated that adult individuals from Obsoletus complex emerged later at cold temperatures in laboratory conditions and *Culicoides* larvae may enter into diapause during cold and unfavourable conditions (Rawlings and Mellor 1994, Lühken *et al*. 2015). The current study showed that *C. obsoletus* only completed its life-cycle at 18ºC and 25ºC. These results coincided with the temperatures in spring during the peak of abundance of the Obsoletus complex in Spain (Ortega *et al*. 1998, 1999, Miranda *et al*. 2004, Cuéllar *et al*. 2008a, present work). Conversely, *C. circumscriptus* completed its development at any temperature and *C. paolae* only finished its life-cycle at 30ºC. This species along with *C. cataneii* showed difficulties in developing at 18ºC in laboratory conditions. The aforementioned biogeographical distributions among the species including the photoperiod (northerly vs southerly species) as well as the long egg hatching period of *C. paolae* and *C. cataneii* were a possible indicator of these difficulties. The low temperature and the artificial substrate may cause a deterrent effect on egg hatching. Hence, variations of temperatures in the laboratory such as swapping the insects from one to other temperature (Lühken *et al*. 2015), could be used in future works to improve the development of immature stages of those species.

The shortest life-cycle was showed by *C. paolae* at 30ºC. This result suggests that this species prefer high temperatures and humidity for breed. *Culicoides paolae* is distributed in Mediterranean climate areas (Estrada *et al*. 2011); so, may be adapted to develop at temperatures and humidity found during summer in Mediterranean climate areas.

Differences between farms in duration within life-cycle stages were probably related with biological differences among populations; however, the duration of each of the four larvae stages and pupa stage were similar among all the taxa studied and the effect of temperature can be observed, especially in the L4 instar larvae which were longer in individuals kept at 18ºC.

The longest life-cycle stage was recorded from the F1 *Culicoides* showing that low temperatures also increased its lifespan. This is the first study where we obtained F1 adults of the vector species *C. obsoletus* and the non-vector species *C. paolae* at 18ºC and 30ºC respectively, underlying the novelty of the current study. F1 adults of *C. obsoletus* reached a maximum of 88 days alive, close to the lifespan recorded from the field-collected gravid females and the values obtained in that of Goffredo *et al*. (2004); thus, demonstrating that the methodology used in the current study was successful for rearing and maintain this species in laboratory conditions.

7.4.5. Percentage of egg hatching, pupation success and adult emergence

In our results, the percentages of egg hatching among all taxa was higher at 25ºC (31.8% ± 34.6%) followed by 18ºC (26.7% ± 31.3%). These rates were lower than the results obtained for a well-adapted colony of *C. nubeculosus* (57.1% - 69.1%) reared at 25ºC (Fahrner and Barthelmess 1988), as well as when compared to the rearing of *C. imicola* conducted at 20ºC, 25ºC and 28ºC in South Africa (Veronesi *et al*. 2009). Lower egg hatching values from our study could be related to differences on the performance at laboratory level of different populations. Differences are higher in particular when compared to laboratory well-adapted colonies, where insects are selected for laboratory breeding generation after generation.

Our results suggest that differences between species in preferred temperatures for development may be related to their global distributions with more northerly species like.

Culicoides obsoletus preferring low temperatures and more southerly species like the ornithophilic species *C. circumscriptus* and *C. paolae* preferred higher temperature regimes for development. In fact, *C. paolae* showed the highest percentage of egg hatching at 30ºC, possibly related to its aforementioned bioecology, showing preferences for Mediterranean temperature regimes.

As well as in the previous chapter, limitations to rearing *C. cataneii* in artificial conditions included its low percentage of egg hatching, the lower percentage of oviposition by females and the lower number of eggs produced per female. This was the second attempt to our knowledge of laboratory rearing of this species; however, better information about its bioecology is required to improve its laboratory rearing.

Culicoides circumscriptus developed best at 25ºC but the egg hatching rates were still lower than the 88.7% obtained by Becker (1960) as found in chapter 6.

Pupation showed also to be different among species according to temperatures and, as found in chapter 6, is considered the most critical point in the life-cycle of *Culicoides* species (Veronesi *et al*. 2009), especially for *C. newsteadi*, *C. cataneii* and *C. imicola*. Percentage of pupation was lower among all tested species at 18ºC due to the high larvae mortality compared to the other temperatures. Other variables affecting the likelihood of pupation are competition for resources or cannibalism (i.e.: larvae predating pupae).

The highest percentage of pupation was recorded by *C. paolae* at 30ºC, showing again its preference for high temperatures. Conversely, no larvae of *C. obsoletus* survived at 30ºC, demonstrating that this species is favoured by mild spring temperatures, as it is shown in its seasonal pattern (Ortega *et al*. 1998, Calvete *et al*. 2008).

The percentage of adults emerged from reared pupae was relatively high in all of the species assayed, demonstrating that is a less sensitive stage of the life-cycle of this genus. Individuals kept at 30ºC showed the highest percentage of adult emergence. F1 adults from *C. circumscriptus* were the only ones emerged at any temperature, making this species suitable for rearing in laboratory conditions. In fact, results for *C. circumscriptus* at 18ºC showed higher percentage of adult emergence than that of Becker (1960) at 17.2ºC, which recorded a percentage of 48.3%.

7.4.6. Pupation

Pupation of *C. obsoletus* was the longest one and occurred during several discrete time periods, instead of being synchronised in a short time. In addition, the number of larvae pupation of *C. obsoletus* was higher and more frequent at 18ºC, demonstrating again the cold adaptation of this species. Conversely, the time for pupation obtained from *C. circumscriptus* and *C. paolae* lasted over one week, being similar to the results from Becker (1960) in case of *C. circumscriptus*. In fact, number of pupae of *C. circumscriptus* was higher at elevated temperatures (except that of *Son Ajaume*). It was also observed that pupation period of *C. circumscriptus* at 18ºC was more evenly distributed than the pupae kept at 25ºC, being related with the deterrent effect of the low temperature on *Culicoides* individuals particularly for more southerly species. The other ornithophilic species *C. paolae* recorded lower number of daily pupae but exhibited a similar pupation period than *C. circumscriptus*, possibly related to the ornithophilic habit of this species.

7.4.7. Larvae growth

In general, 25ºC was the most suitable temperature for larvae development and lower temperatures such as 18ºC reduced larvae growth among all taxa included in the current study. Species such as *C. circumscriptus* (the species of largest body size) and *C. paolae* grew faster than Obsoletus complex at any temperature, suggesting faster daily rates of development related to their higher metabolic activity. It is well known that temperature clearly plays an important role in the larvae development inducing diapause periods during low temperatures (Rawlings and Mellor 1994, Lühken *et al*. 2015).

7.4.8. Sex ratio

The ornithophilic species *C. circumscriptus* and *C. paolae* had similar sex ratio supporting the results from Becker (1960) which obtained an average of 51% *C. circumscriptus* females. As well as other haematophagous insects such as the yellow fever mosquito *Aedes aegypti* (L.) (Mohammed and Chadee 2011), temperature may play also an important role in sex determination of *Culicoides*, the mechanisms for which remains unclear. Males were predominant in the case of *C. obsoletus,* which reached a 100% of males at 18ºC. Higher mortality among female larvae or limitations in the feeding method to support *C. obsoletus* female development compare to males are possible explanations pointed out by Veronesi *et al*. (2009) and Nevill (1967).

7.5. Conclusions

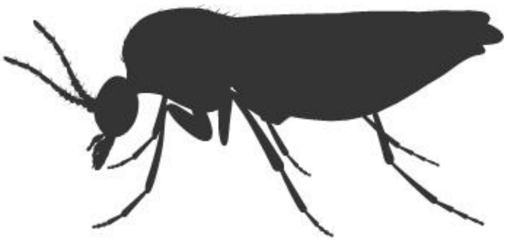
This study has contributed to the understanding of the basic bionomics at different temperatures of farm associated species of *Culicoides* in Spain. In fact, this is the first study where *C. obsoletus* and *C. paolae* completed their life-cycle at these temperatures in laboratory conditions; *C. obsoletus* at 18 and 25ºC and *C. paolae* at 30ºC.

All species kept 18ºC recorded the longest lifespans and oviposition periods and Obsoletus complex species clearly showed preference for low temperatures indicating its adaption to cold temperatures. Conversely, *C. circumscriptus* and *C. paolae* prefer higher temperatures for its development which indicates warm adaptation.

Hence, the vector species *C. obsoletus* and the non- vector species *C. circumscriptus* and *C. paolae* are the most promising species to be reared in laboratory conditions, due to its high oviposition percentages, lifespan, percentages of egg hatching, pupation and adult emergence, in addition to temperature adaptability in case of *C. circumscriptus*. In fact, this species is able to transmit pathogens such as *Plasmodium* and *Leishmania* in field conditions (Ferraguti *et al*. 2013, Slama *et al*. 2014) and thus is of high priority for laboratory rearing.

These results must be considered in terms of BTV prevalence, since the climatic fluctuations in temperate regions such as Spain are large and the total transmission potential of an infected vector is maximized at intermediate temperatures (Brand and Keeling 2017). However, we are far to find the best requirements of the European BTV vector species *C. imicola* and *C. newsteadi* for laboratory rearing or even to stablish a colony in the laboratory. Oviposition and larvae substrates, material of cages for keeping the gravid females, oscillations in temperatures and humidity are subjects to improve to check the preferences of *Culicoides* species for breed. Mating conditions of these insects is one of the major obstacles for understanding the rearing requirements (Jamnback 1961, Kettle 1977, Veronesi *et al*. 2009). Some species such as *C. obsoletus* mate in swarms (Kettle 1995, Kirkeby 2018) but the details of this behaviour in vector *Culicoides* species remains unknown (Blackwell *et al*. 1992, González *et al*. 2017). Several methods were used in Becker (1960) to persuade *C. circumscriptus* to mate in laboratory conditions but without success; suggesting that this species may mate while the insects are at rest. These particularities highlighted that the main obstacle is the refusal by the adults to mate in captivity; so, further studies including mating of different species could be interesting.

8. Recapitulación



8. Recapitulación

La llegada y expansión de enfermedades de origen tropical o subtropical transmitidas por insectos vectores causan importantes problemas tanto médico-veterinarios como económicos, por lo tanto, el conocimiento de la biología y ecología de esos vectores es crucial para comprender la transmisión de arbovirosis y otros patógenos. Gracias a esos conocimientos podemos actuar en el momento preciso poniendo en marcha las medidas necesarias para su control y así evitar una posible epidemia o minimizar sus efectos.

En el presente trabajo se han realizado cinco estudios diferentes relacionados con la fenología y bionomía básica de dípteros del género *Culicoides*, principales vectores del VLA en España:

- Un análisis del patrón estacional de hembras de *Culicoides* nulíparas y paras en España usando un eje latitudinal.
- La determinación de los factores ambientales que afectan a la fenología y abundancia de *Culicoides* vectores del VLA en España mediante modelos generalizados lineales mixtos.
- El uso de modelos de análisis de trayectorias (*Path Analysis models*) con el fin de determinar los parámetros ambientales que afectan directamente o indirectamente a la fenología de *Culicoides* nulíparas y paras.
- Un estudio en condiciones de laboratorio de la bionomía básica de *Culicoides* vectores y no vectores asociados a granjas en la isla de Mallorca (Islas Baleares), incluyendo un ensayo preliminar a la alimentación artificial de *C. obsoletus* con sangre de vacuno.
- El uso de distintas temperaturas para comparar la bionomía básica del complejo Obsoletus en condiciones de laboratorio además de otras especies asociadas a explotaciones ganaderas.

En el primer estudio se analizó por primera vez la dinámica poblacional de las hembras de *Culicoides* paras (PF) durante tres años consecutivos en España, ya que se trata de la única fracción que puede transmitir el VLA. Se observó que durante los meses de verano más de la mitad de la población se encuentra en ese estado gonotrófico. En el caso de las especies del complejo Obsoletus, la actividad de PF continúa incluso en invierno, sobretodo en el Norte de la península donde es más abundante. De hecho, la última semana de Diciembre fue el único momento considerado como periodo estacionalmente libre de vectores (SVFP) en España entre los años 2008 y 2010. En el análisis latitudinal vimos que las provincias situadas más al Norte tuvieron un SVFP más largo. La especie *C. imicola* no se encontró en provincias por encima de Toledo, de hecho, esta provincia presentó los índices de abundancia más elevados para esta especie junto con otras dos especies vectores *C. newsteadi* y *C. pulicaris*, las cuales parecen centrar su actividad en zonas del interior de la península Ibérica. Por lo tanto, se debería realizar un mayor esfuerzo en el manejo de la enfermedad en esa provincia. Estos resultados sugieren que sería interesante un análisis usando no solo parámetros climáticos y topográficos, sino también usos de suelo, densidad de hospedadores, etc. con el objetivo de determinar con más detalle los factores de riesgo de transmisión del VLA.

Los resultados del segundo estudio han permitido determinar la abundancia y los factores ambientales que determinan el inicio, el fin y la diapausa de la actividad estacional de las cuatro especies de *Culicoides* vectores del VLA (complejo Obsoletus, *C. imicola*, *C. newsteadi* y *C. pulicaris*). Se observó que cada especie se ve afectada por variables ambientales distintas, lo cual nos ayuda a entender la biología y el comportamiento de estos insectos. El complejo Obsoletus presenta una distribución más amplia y las primeras del año en aparecer en España mientras que *C. imicola* es la que presenta mayor volumen de capturas y la más tardía en aparecer. De todas las variables incluidas en los modelos, el clima (temperatura y precipitación) y la topografía fueron las que tuvieron una mayor influencia en la fenología del complejo Obsoletus y *C. imicola*. La abundancia de ganado y el fotoperiodo también tuvieron un papel importante en Obsoletus complex mientras que la fenología de *C. newsteadi* viene también determinada por otros factores como la cobertura vegetal del suelo. En el caso de *C. pulicaris*, la única variable significativa fue la temperatura en invierno, lo cual sugiere que este tipo de modelos no acaba de encajar con esta especie debido posiblemente a su distribución o al bajo volumen de capturas registrado para esta especie. Estos resultados pueden servir para determinar las zonas con circunstancias ambientales adecuadas para el desarrollo de cada una de estas especies en particular, lo cual deberían tenerse en cuenta a la hora de seleccionar las zonas de vigilancia de *Culicoides* en España a la hora de detectar y prevenir posibles infecciones de patógenos transmitidos por estos insectos

Los análisis de trayectorias (PA) del tercer estudio nos han ayudado a analizar con más detalle los datos usados durante el estudio anterior. En ellos se han determinado cuáles son las variables ambientales que afectan solo a la fenología y cuáles afectan indirectamente a la fenología a través de un aumento de las hembras de *Culicoides*. Además, se separaron las hembras nulíparas y paras para también determinar qué variables ambientales afectan a un estado gonotrófico o a otro. Se observó que la abundancia de hembras de *C. imicola* se ve significativamente afectada por la vegetación esclerófila típica del Mediterráneo, afectando indirectamente a la fenología de las hembras nulíparas de esta especie. Por otro lado, la cobertura del suelo, la topografía y el fotoperiodo afectaron exclusivamente a la fase para de las hembras de Obsoletus complex. Las otras dos especies, *C. pulicaris* y *C. newsteadi* se vieron afectadas por pocas variables, de hecho, los resultados no mostraron ninguna variable significativa en el final del periodo de actividad de las hembras adultas de estas especies. De hecho, la única variable significativa para *C. newsteadi* fue la abundancia de *Culicoides* hembras, mientras que *C. pulicaris* se vio afectado principalmente por el fotoperiodo y las temperaturas en invierno, igual que en el estudio anterior. Estos resultados nos han ayudado a entender mejor la fenología de adultos de estas especies gracias a variables ambientales. Esas varíales deberían tenerse en cuenta para determinar el SVFP en España. De hecho, este tipo de estudio podría usarse con otros insectos vectores como los mosquitos. Además, en futuros estudios se podrían añadir más variables como la temperatura y la infracción del suelo para mejorar los resultados obtenidos.

En el cuarto estudio nos enfocamos en parámetros bionómicos de fases inmaduras y adultos de diferentes especies de *Culicoides* ya que se trata de un campo poco estudiado. Para ello, se obtuvieron hembras grávidas de *Culicoides* obtenidas del campo y se alimentaron hembras nulíparas de *C. obsoletus* artificialmente con sangre de vacuno en el laboratorio. Un total de seis especies (*C. newsteadi*, *C. cataneii*, *C. paolae*, *C. circumscriptus*, Obsoletus complex y *C. imicola*) consiguieron completar su ciclo a 25ºC en el laboratorio. De hecho, esta fue la primera vez que se consigue completar el ciclo en condiciones de laboratorio de *C. newsteadi*, C. *cataneii*, *C. paolae* y Obsoletus complex y de poblaciones Mediterráneas de *C. circumscriptus* y *C. imicola*. Se observó que hubo variaciones en la bionomía dependiendo de distintos factores como el método de alimentación con sangre, los sustratos de oviposición y desarrollo larvario o la temperatura. Además, algunas especies presentaron dificultades en alguna fase de su desarrollo. De hecho, los resultaros mostraron que la fase larvaria es el punto más crítico debido al bajo porcentaje de pupado, sin embargo, las dos especies ornitofílicas, *C. circumscriptus* y *C. paolae*, fueron las especies que mejor se adaptaron. Por otro lado, las especies vectores *C. obsoletus* y *C. imicola* presentaron valores de esperanza de vida de adultos elevados, aumentando el periodo de transmisión de patógenos como el VLA. Igual que en otros estudios, la tasa de alimentación con sangre fue baja (6.25%). Algunos parámetros como el porcentaje de oviposición o la supervivencia de los adultos fueron mayores en la progenie obtenida gracias a las hembras alimentadas artificialmente, sin embargo, es necesaria una mayor tasa de alimentación artificial con el fin de obtener un mayor número de hembras alimentadas. Por lo tanto, deben considerarse futuros ensayos con diferentes métodos de alimentación a fin de conseguir mejores resultados. En general, los datos obtenidos nos ayudaron a entender mejor los aspectos bionómicos de las especies estudiadas. La sensibilidad y duración de la fase L4 de estos insectos sugiere que deberían focalizarse métodos de control sobre ese estado larvario. Por otro lado, solo existen dos especies de *Culicoides* en el mundo con las cuales se ha conseguido crear una colonia (*C. nubeculosus* y *C. sonorensis*), sin embargo, estamos lejos de poder establecer una colonia en el laboratorio de especies vectores como *C. obsoletus* o *C. imicola* ya que sus condiciones de cópula son totalmente desconocidos. En futuros estudios se podría incluir este parámetro, así como un ensayo utilizando diferentes sustratos de oviposición y desarrollo larvario o incluso diferentes temperaturas.

En el último capítulo se estudió la bionomía a tres temperaturas diferentes (18ºC, 25ºC y 30ºC) a partir de hembras grávidas de especies vectores y no vectores obtenidas en el campo de dos poblaciones diferentes. Los resultados obtenidos mostraron que por una parte los individuos mantenidos a temperaturas bajas aumentan el tiempo de desarrollo de las fases inmaduras y de los adultos, mientras que temperaturas más altas aumentaron el porcentaje de pupado y emergencia de los adultos. Se obtuvieron huevos de cuatro especies diferentes: *C. obsoletus*, *C. circumscriptus*, *C. paolae* y *C. cataneii*. La especie *C. circumscriptus* consiguió terminar su ciclo a las tres temperaturas estudiadas mientras que *C. obsoletus* solo lo terminó a 18ºC y 25ºC, *C. paolae* a 30ºC y *C. cataneii* a ninguna de ellas. Estos resultados sugirieron que la especie *C. obsoletus* parece estar mejor adaptada a temperaturas bajas mostrando mayores tasas de supervivencia y oviposición a 18ºC demostrando que se trata de una especie adaptada a zonas con temperaturas más bajas como el Norte de Europa. Por otro lado, las especies ornitofílicas *C. circumscriptus* y *C. paolae*, las cuales ya se comentó que eran buenas candidatas para su cría en cautividad, parecen estar mejor adaptadas a temperaturas superiores, ya que presentaron un mayor porcentaje de oviposición y número de huevos a 25ºC y 30ºC. En futuros estudios se pueden utilizar diferentes tipos de sustratos y variaciones de temperatura o de humedad para conseguir un mayor porcentaje de oviposición o incluso obtener huevos de otras especies que no consiguieron ovipositar como *C. jumineri*, *C. puncticollis*, *C. parroti* o *C. brunnicans*. Gracias a los datos que sabemos sobre la bionomía de *Culicoides* vectores y no-vectores, podemos predecir cómo puede variar el desarrollo de estos insectos en zonas templadas como España donde se registran grandes fluctuaciones de temperatura, por lo tanto, deben tenerse en cuenta a la hora de evaluar la prevalencia de VLA.

9. Conclusions

1. The abundance of *C. imicola* increased from North to South axis in Spain. In fact, the Northern provinces were absent of this species during the whole year. Conversely, Obsoletus complex abundance increased from South to North.

2. The species *C. newsteadi* and *C. pulicaris* were also present in all localities but less abundant, showing higher abundance in inland zones such as Toledo province, overlapping with the other vector species *C. imicola*.

3. During the National Surveillance program in Spain, the species from Obsoletus complex were the most prevalent across the whole country between 2005 and 2010 and the ones which appear earlier in the year. On the other hand, *C. imicola* recorded the highest number of individuals collected and was the latest species appearing in the year.

4. The temperature in winter and the elevation above the sea level were the most explanatory variable in the seasonal models.

5. *Culicoides imicola* phenology was favoured by low altitude sites and path analysis models showed that schlerophyllous vegetation make an indirect effect on the phenology of *C. imicola* nulliparous females via increasing female abundance.

6. Obsoletus complex also preferred low altitude sites with long hot days in spring and autumn in addition to sites with high precipitation in spring and autumn, including high density of *Culicoides* females and bovine livestock. Conversely, Path analysis showed that schlerophyllous vegetation and low number of daylight hours during April decreases the activity period of Obsoletus complex parous females.

7. *Culicoides newsteadi* was the species that were affected with more variables. Longer activity periods of that species were observed in sites with high density of sheep, high temperatures and precipitation in winter, high temperatures in autumn, Agro-forestry areas and sites with high schlerophyllous vegetation areas. Conversely, Path analysis

of *C. newsteadi* showed that phenology of this species was only strongly affected by *Culicoides* females abundance.

8. The species *C. pulicaris* was the one with shorter activity period. The models seem not to fit properly on that species, being only sites with hot winters and low number of daylight hours in November the significant variables showing longer activity of *C. pulicaris*.

9. The ornithophilic species *C. circumscriptus* and *C. paolae* are the most promising species to be reared in laboratory conditions due to its high oviposition percentages, high number of females emerged, long lifespan and fast life-cycle; meanwhile, *C. obsoletus*, *C. imicola*, *C. newsteadi* and *C. cataneii* showed limitations on oviposition and larvae development under laboratory conditions at 25ºC.

10. Despite the lower artificial feeding rate recorded from *C. obsoletus*, several parameters such as percentage of oviposition, lifespan and time to egg hatching were higher in *C. obsoletus* artificially engorged females; whereas field *C. obsoletus* showed higher percentage of pupation and higher total number of eggs, pupae and females emerged.

11. Low temperature increased the lifespan, time to oviposition and time of larvae development of the *Culicoides* species whereas high temperature increased the number of eggs laid and the percentage of pupation and adult emergence.

12. Obsoletus complex species were clearly adapted to low temperatures. Individuals kept at 18ºC recorded higher lifespans and percentage of oviposition; meanwhile, *C. circumscriptus* and *C. paolae* preferred higher temperatures.

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11. Formación predoctoral

11.1. Publicaciones relacionadas con la presente tesis

Barceló, C. & Miranda, M. A. 2017. Bionomics of livestock-associated *Culicoides* (biting midge) bluetongue virus vectors under laboratory conditions. Medical and Veterinary Entomology, 32 (2), 216-225.

Del Río, R., **Barceló, C**., Lucientes, J., & Miranda, M. A. 2014. Detrimental effect of cypermethrin treated nets on *Culicoides* populations (Diptera; Ceratopogonidae) and non-targeted fauna in livestock farms. Veterinary parasitology, 199(3), 230-234.

Del Rio, R., Venail, R., Calvete, C., **Barceló, C**., Baldet, T., Lucientes, J., & Miranda, M. A. 2014. Sensitivity of *Culicoides obsoletus* (Meigen) (Diptera: Ceratopogonidae) to deltamethrin determined by an adapted WHO standard susceptibility test. Parasitology, 141(4), 542-546

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Cuéllar, A. C., L. J. Kjær, C. Kirkeby, H. Skovgard, S. A. Nielsen, A. Stockmarr, G. Anderson, A. Lindstrom, J. Chirico, R. Lühken, S. Steinke, E. Kiel, J. Gethmann, F. J. Conraths, M. Larska, I. Hamnes, S. Sviland, P. Hopp, K. Brugger, F. Rubel, T. Balenghien, C. Garros, I. Rakotoarivony, X. Allène, J. Lhoir, D. Chavernac, J. C. Delécolle, B. Mathieu, D. Delécolle, M.-L. Setier-Rio, R. Venail, B. Scheid1, M. Á. Miranda, **C. Barceló**, J. Lucientes, R. Estrada, A. Mathis, W. Tack, and R. Bødker. 2018. Spatial and temporal variation in the abundance of *Culicoides* biting midges (Diptera: Ceratopogonidae) in nine European countries. Parasites & Vectors. 11, 112.

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11.2. Contribuciones a congresos

11.2.1. Comunicaciones orales

Barceló, C., Estrada, R., Lucientes, J., Miranda, M.A., Searle, K.R., Purse, B. V. 2013. Seasonal activity and abundance of biting midges (*Culicoides* spp.), vector species of bluetongue virus in Spain. EDENext meeting 2013, Barcelona. Spain.

Barceló, C., Estrada, R., Lucientes, J., Miranda, M.A., Searle, K.R., Purse, B. V. 2014. How do environmental factors determine the adult seasonality of *Culicoides* spp.? A modelling approach. EDENext meeting 2014, Rovaniemi. Finland.

Del Río, R., **Barceló, C**., Venail, R., Lucientes, J., Miranda, M.A. 2014. Sensitivity of *Culicoides obsoletus* to cypermethrin in laboratory conditions. EDENext meeting 2014, Rovaniemi. Finland.

Miranda, M.A., **Barceló, C**., Del Río, R. 2014. Effect of deltamethrin treated nets on *Culicoides* (Diptera; Ceratopogonidae) population and non-targeted fauna in livestock farm in the Balearic Islands (Spain). 19th E-SOVE conference, Thessaloniki. Greece.

Miranda, M.A., Lucientes, J., **Barceló, C**. Control of *Culicoide*s (Diptera; Ceratopogonidae) farm associated species: reality and perspectives. 2015. 7th European Mosquito Control Association Workshop (EMCA 2015), Valencia. Spain.

Miranda, MA., **Barceló, C**., Del Río, R., Lucientes, J. 2015. The use of insecticide impregnated nets against *Culicoide*s spp.: state of the art and progress in the project EDENext. GERI 2015, Heraklion. Greece.

Barceló, C., Miranda, M.A. 2016. Study of the bionomics of the Obsoletus complex and other livestock associated *Culicoides* at different temperatures in laboratory conditions. 20th E-SOVE conference, Lisbon. Portugal

Miranda, M.A., **Barceló, C**., Borràs, D., Gerry, A.C. Understanding the role of host emitted semiochemicals on *Culicoides* (Diptera; Ceratopogonidae) behavior. 20th E-SOVE conference. Lisbon, Portugal.

Miranda, M.A., Borràs, D., García, R.., Ruiz, M., Paredes-Esquivel, C., **Barceló, C**., Gumá, M., Ramos, M. 2016. Implementation of entomological surveillance around imported cases of arbovirus diseases in the Balearic Islands (Spain). . 20th E-SOVE conference, Lisbon. Portugal

Miranda, M.A., **Barceló, C**. 2016. Exploring semiochemicals for improving monitoring and control of *Culicoides* spp. XXV International Congress of Entomology (ICE), Orlando. EEUU

Cuéllar, A.C., Kjær, L.J., Skovgaard, H., Nielsen, S.A., Stockmarr, A., Anderson, G., Lindström, A., Chirico, J., Lilja, T., Lühken, R., Steinke, S., Kiel, E., Larska, M., Hamnes, S. I., Sviland, S., Hopp, .P, Brugger, K., Rubel, F., Balenghien, T., Garros, C., Rakotoarivony, I., Allene, X., Lhoir, J., Delecolle, J. C., Mathieu, B., Delecolle, D., Setier-Rio, M.L., Venail, R., Scheid, B., Miranda, M. A.; **Barceló, C**., Lucientes, J., Estrada, R., Wesley, T., Mathis, A., Bødker, R. 2017. Big data - modelling of midges in Europa using machine learning techniques and satellite imagery. NKVet Symposium 2017, Oslo. Norwegian.

Barceló, C., Miranda, M.A. 2017. From egg to adult. How do *Culicoides* early stages live under laboratory conditions? 7th SOVE international congress, Palma. Spain.

Barceló, C., Miranda, M.A. 2018. De huevo a adulto. ¿Cómo viven las fases inmaduras de *Culicoides* en condiciones de laboratorio? I Congreso Nacional de investigadores en formación, Granada. Spain.

Barceló, C., Estrada, R., Lucientes, J., Miranda, M.A. 2018. The latitude determines the *Culicoides* species composition and its seasonal pattern in mainland Spain. 21th E-SOVE conference, Palermo. Italy.

Barceló, C., Del Río, R., Miranda, M.A. 2018. Temperature dependent lifespan and emergence traits of *Culicoides* Latreille species (Ceratopogonidae) under laboratory conditions. 9th International Dipterology Congress, Windhoek. Namibia.

Miranda, M.A., **Barceló, C**., Anchor, C., O' Hara, M., Cohnstaedt, L. 2018. Species composition of potential epizootic hemorrhagic disease vector species in North Illinois, U.S.A. 9th International Dipterology Congress, Windhoek. Namibia.

Miranda, M.A., **Barceló, C**., Borràs, D., Miquel, M., Monerris, M,; Del Río, R., Paredes-Esquivel, C. 2018. Twenty-five years of the study of Diptera of economic and health importance in the Balearic Islands, Spain. 9th International Dipterology Congress, Windhoek. Namibia.

11.2.2. Posters

Miranda, M.A., Del Río, R., Miquel, M., Paredes-Esquivel, C., **Barceló, C**., Lucientes, J. 2012. Evaluation of the efficacy of deltamethrin treated nets in the control of *Culicoides* spp. in field conditions. 18th E-SOVE conference, Montpelier, France.

Barceló, C., Del Río, R., Borràs, D., Miranda, M.A., Estrada, R., Lucientes, J., Searle, K.R., Purse, B.V. 2013. Modelling the seasonal activity and abundance of biting midges (*Culicoides* spp.), vector species of bluetongue virus in Spain. 6th SOVE international congress, California. USA.

Miranda, M.A., Del Río, R.; **Barceló, C**., Lucientes, J. 2013. Detrimental effect of cypermethrin treated nets on Culicoides populations (Diptera; Ceratopogonidae) and non-targeted fauna in livestock farms. 6th SOVE international congress, California. USA.

Barceló, C., Miranda, M.A. 2014. Study of the bionomics of farm associated *Culicoides* species in laboratory conditions. . IV international congress of bluetongue and related orbiviruses, Rome. Italy.

Barceló, C., Miranda, M.A. 2015. Bionomics of Obsoletus complex species and other livestock associated *Culicoides* in laboratory conditions. GERI 2015, Heraklion, Greece.

Miranda, MA., **Barceló, C**., Del Río, R., Lucientes, J. 2015. The use of insecticide impregnated nets against *Culicoides* spp.: state of the art and progress in the project EDENext. GERI 2015, Heraklion, Greece.

Barceló, C., Estrada, R., Lucientes, J., Miranda, M.A. 2018. Seasonal patterns of *Culicoides* nulliparous and parous females at different latitudes in Spain between 2008 and 2010. InnovSur Conference, Montpellier. France.

11.3. Estancias en centros de investigación

CEH (Center for Ecology and Hydrology), Penicuik (Edimburgo, Escocia): Nov.- dic. 2012 / Enero - febr. 2013 y febrero 2014.

CEH (Centre for Ecology and Hydrology), Wallingford (Oxfordshire, Inglaterra): Marzo 2014

11.4. Cursos recibidos

Control parasitológico integrado en animales. Febrero 2015. COVIB y UIB.

Jornada técnica. Actualización en plagas urbanas y sus vectores: Un problema sanitario. Noviembre 2016. Ámbito: Nacional. COVIB.

Curso de formación sobre enfermedades tropicales desatendidas: De la oscuridad a la penumbra. La lucha contra la malaria y las enfermedades olvidadas del siglo XXI. Mayo 2017. UIB-OCDS.

MOOC course (online) - Medical entomology - Insect vectors and transmission of pathogens (2019). Institute Pasteur

Curso de entomología sanitaria y control de vectores. Julio 2019. Universidad de Zaragoza.

“Hay quienes se gradúan a los 22 y tienen trabajo a los 27. Alguien ya tenía un postgrado a los 25 pero murió a los 50. Otros no estudiaron carrera y son millonarios mientras que algunos tienen un máster pero no capacidad de hacer dinero. Hay quienes tienen pareja y aman a otra, hay quienes se aman y no son nada.

Pues todos en este mundo viven de acuerdo a su propio tiempo. Las personas que te rodean pueden parecer ir delante de ti, pero todos están corriendo su propia carrera en su propio tiempo. No los envidies, están en su vida y tú estás en la tuya. Así que relájate. No has llegado tarde. No has llegado temprano. Todo está justo a tiempo.”

Autor desconocido

Portada: Carolina Amigo Seguí

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