Universitat Autònoma de Barcelona

THE MICROBIOME AND VECTOR MOSQUITOES: New Insights for Malaria and Arbovirus Control and Surveillance

Lotty Birnberg Yerovi

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Universitat Autònoma de Barcelona

Facultat de Veterinària

Departament de Sanitat i d'Anatomia Animals

THE MICROBIOME AND VECTOR MOSQUITOES: New Insights for Malaria and Arbovirus Control and Surveillance

PhD Thesis

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(Fragment, original illustration: Abraham Birnberg, 2021)

THE MICROBIOME AND VECTOR MOSQUITOES: New Insights for Malaria and Arbovirus Control and Surveillance

Tesis doctoral presentada por **Lotty Birnberg Yerovi** para acceder al grado de Doctora en el marco del programa de Doctorado en *Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona*, bajo la dirección de **Núria Busquets i Martí** y la tutoría de **Francesc Accensi i Alemany.**

Bellaterra, 2022

La Dra. **Núria Busquets i Martí**, investigadora del *Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal* (IRTA-CReSA) como directora y Dr. **Francesc Accensi i Alemany**, profesor titular del *Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universidad Autónoma de Barcelona* e investigador adscrito al IRTA-CReSA como tutor de la presente tesis

Certifican:

Que los trabajos de investigación incluidos en la memoria de tesis doctoral "***THE MICROBIOME AND VECTOR MOSQUITOES: New Insights for Malaria and Arbovirus Control and Surveillance***", presentados por la doctoranda **Lotty Birnberg Yerovi** para la obtención del Grado de Doctor en *Medicina i Sanitat Animals* se han realizado bajo su dirección y tutoría. Por consiguiente, autorizan su presentación a fin de ser evaluados por la comisión correspondiente.

Para que así conste y a efectos oportunos, firman el presente certificado.

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Lotty Birnberg Yerovi

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Portada e ilustraciones internas originales de Abraham Birnberg (@xawaabraham) protegidas por leyes de derecho de autor,

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LIST OF ABBREVIATIONS

aa, amino-acid
ANOVA, analysis of variance
ATP, adenosine 5'-triphosphate
BATV, Batai virus
BFV, Barmah Forest virus
BIC, Bayesian information criterion
BLAST, basic local alignment search tool
bp, base-pairs
BSL2, Biosafety Level 2
BSL3, Biosafety Level 3
Bti, *Bacillus thuringiensis israelensis*
CBPV, chronic bee paralysis virus
cDNA, complementary deoxynucleic acid
CFAV, cell fusing agent virus
CHIKV, chikungunya virus
CO_2, carbon dioxide
CPE, cytopathic effect
Cq, quantification cycle
CuTLV, *Culex Tymoviridae*-like virus
CxFV, Culex flavivirus
DENV, dengue virus
DIR, disseminated infection rate
DMEM, Dulbecco's modified Eagle medium
DNA, deoxyribonucleic acid
dpe, days post-exposure
dpi, days post-inoculation
dsDNA, double-stranded DNA genomes
dsRNA, double-stranded RNA genomes
E, newly emerged females
EID, emerging infectious disease F0, wild-caught females
EIP, extrinsic incubation period
F2, second laboratory generation
F4, fourth laboratory generation
F6, sixth laboratory generation
F10, tenth laboratory generation
FBS, fetal bovine serum

FDR, false-discovery rate
FEF, fully engorged females
FR, feeding rate
FTA, Flinders Technology Associates
GLM, generalized linear model
HOUV, Houston virus
HR, hatching rate
IR, infection rate
IRTA-CReSA, Institut de Recerca i Tecnologies Agroalimentaries - Centre de Recerca en Sanitat Animal
ISFV, insect-specific flavivirus
ISV, insect-specific viruses
ITS, internal transcribed spacer
JCV, Jamestone Canyon virus
L, larvae
LCBD, local contribution to beta diversity
LMR, larval mortality rate
log_2FC, logarithm 2 of the relative change
LSU, large subunit
MBFV, mosquito-borne flaviviruses
MBD, mosquito-borne diseases
MCDM, multicriteria decision-making
MDA, multiple displacement amplification
MEB, midgut escape barrier
MIB, midgut infection barrier
ML, maximum likelihood
ml, mililiter
MPM, meconial peritrophic matrix
na, data not available
NCBI, National Center for Biotechnology Information
NDiV, Nam Dinh virus
NGS, next generation sequencing
NIAID, National Institute of Allergy and Infectious Diseases
NS, nonstrustural
nt, nucleotides
OIE, World Organization for Animal Health
PBS, phosphate-buffered saline
PCoA, principal coordinate analysis
PCLV, Phasi Charoen-like virus
PCR, polymerase chain reaction

PERMANOVA, permutational multivariate analysis of variance
PFU/ml, plaque-forming units per milliliter
ORF, open reading frame
OTU, operational taxonomic unit
RdRp, RNA-dependent RNA polymerase gene
RH, relative humidity
RNAi, RNA interference
RNase A, ribonuclease A
rRNA, ribosomal ribonucleic acid
RRV, Ross River virus
RT-nPCR, reverse transcription nested polymerase chain reaction
RT-PCR, reverse transcription polymerase chain reaction
RT-qPCR, reverse transcription quantitative polymerase chain reaction
RVF, Rift Valley fever
RVFV, Rift Valley fever phlebovirus
SARS-CoV-2, severe acute respiratory syndrome coronavirus 2
SB, salivary gland barrier
SINDV, Sindbis virus
SPF, specific pathogen-free
SRA, sequence read archive
ssDNA, single-stranded DNA genomes
SSHV, Snowshoe hare virus
ssRNA-, single-stranded negative sense RNA genomes
ssRNA+, single-stranded positive-sense RNA genomes
SSU, small subunit
T0, initial time point, sylvan environment
TAHV, Tahyna virus
$TCID_{50}$/ml, 50% tissue culture infective dose per milliliter
TE, transmission efficiency
TR, transmission rate
UK, United Kingdom
USUV, Usutu virus
VC, vector competence
W, natural breeding water
WBDV, Wiesbiden virus
WHO, World Health Organization
WNV, West Nile virus
ZIKV, Zika virus

ABSTRACT

The threat of the resurgence or introduction of mosquito-borne diseases, such as malaria and Rift Valley fever, into the European continent has awakened new interests in studying the microbiome associated to autochthonous mosquitoes for better understanding mosquito-pathogen interaction and developing ecologically adequate vector surveillance and control tools. Consequently, this thesis aimed to i) explore the microbiota of *Anopheles atroparvus*, a vector involved in malaria transmission in Europe, ii) assess the influence of insect-specific flaviviruses on the vector competence of European *Culex pipiens* and *Aedes vexans* for the transmission of RVFV, and iii) apply metagenomics on FTA cards as a new approach for virus detection and arbovirus surveillance.

In the first chapter, a laboratory colony of *An. atroparvus* from the Ebro Delta was established, and its rearing protocol updated. Sequencing of the bacterial 16S rRNA gene showed that the breeding environment, physiology and foraging habits influenced the microbiota of field-caught and laboratory-colonized mosquitoes. Diversity analyses showed inter-sample variation among sylvan developmental stages and a diversity decline in adult females after ten-laboratory generations. Nonetheless, a significant fraction of the microbiota was conserved from wild-caught specimens until the tenth laboratory-generation. Environmentally acquired Gram-negative proteobacteria dominated the microbiota of this anopheles population, among which, *Pseudomonas*, *Asaia* and *Serratia* were identified as potential candidates to be studied for local vector control.

To assess the influence of ISFVs on the vector competence for the transmission of RVFV (Chapter 2), the infection of Culex flavivirus (CxFV) was first studied in *Cx. pipiens* by oral exposure and intrathoracic inoculations.

CxFV infected *Cx. pipiens* after intrathoracic inoculations but not after oral exposure. Then, RVFV vector competence assays in co-infection with CxFV and a mosquito-flavivirus of natural circulation were conducted in *Cx. pipiens* and *Ae. vexans* respectively. Both Catalonian species showed to be competent vectors for RVFV after oral exposure. CxFV nor RVFV interfered with each other´s infection, while, naturally infecting mosquito-flavivirus, although it does not avoided transmission, modulated RVFV infection susceptibility in *Ae. vexans*, suggesting its potential use as bio-agent for preventing RVFV transmission.

Finally, to assess new alternatives for circulating viruses' detection and arboviral surveillance (Chapter 3), next generation sequencing was applied on honey-baited FTA cards that were exposed to field-captured mosquitoes during entomological surveys. Arthropod- and plant-infecting viruses were identified on FTAs and near-complete viral genomes were obtained suggesting good quality preservation of viral RNAs. To confirm the presence of mosquito-associated viruses in the captured specimens, mosquito pools were screened using reverse-transcription PCRs and species-specific primers designed from the sequences obtained from the FTAs. Viruses related to *Alphamesonivirus, Quaranjavirus* and unclassified *Bunyavirales* were detected in Catalonian mosquitoes. These findings constitute the first distribution record of these insect-specific viruses in European mosquitoes. Detecting ISVs in mosquitoes' saliva in field conditions demonstrate the feasibility of this approach to monitor the transmissible fraction of the mosquitoes' virome and its suitability for arbovirus surveillance.

Overall, the present work contributes with valuable information for better understanding the factors behind the structure of the microbiome of local vector mosquitoes, its potential influence in vector competence, and provides a new approach to complement arbovirus surveillance in susceptible areas and to detect circulating and new potentially pathogenic viruses.

RESUMEN

En Europa, la amenaza del resurgimiento e introducción de enfermedades transmitidas por mosquitos, como la malaria y la fiebre del Valle de Rift, ha despertado un nuevo interés en el estudio del microbioma asociado con mosquitos autóctonos para un mejor entendimiento de las interacciones mosquito-patógeno con el fin de desarrollar herramientas de vigilancia vectorial y control ecológicamente más adecuadas. Consecuentemente, la presente tesis se enfocó en: i) explorar la microbiota de *Anopheles atroparvus*, ii) evaluar la influencia de flavivirus insecto-específico en la competencia vectorial de *Culex pipiens* y *Aedes vexans* de Europa para la transmisión de RVFV, y iii) Aplicar metagenómica en tarjetas FTA como un nuevo método para la detección de virus y vigilancia de arbovirus.

En el primer capítulo, se estableció una colonia de laboratorio de *An. atroparvus* del Delta del Ebro y se actualizó su protocolo de cría. El secuenciamiento del gen bacteriano 16S rRNA mostró que el ambiente de cría, fisiología y hábitos de forrajeo influyeron en la microbiota de mosquitos tanto de campo como de laboratorio. Adicionalmente, los análisis de diversidad mostraron variación entre los diferentes estadios de desarrollo silvestres y un declive de diversidad en hembras de la décima generación de laboratorio, sin embargo, una fracción significativa de la microbiota de hembras silvestres fue conservada. Finalmente, proteobacterias Gram-negativas predominaron en la microbiota de *An. atroparvus*, entre las cuales *Pseudomonas*, *Asaia* y *Serratia* fueron identificadas como candidatas potenciales para control vectorial local.

Para evaluar la influencia de ISFVs en la competencia vectorial frente a la transmisión de RVFV (Capítulo 2), primero se estudió la infección de Culex flavivirus (CxFV) en *Cx. pipiens* a través de exposición oral e inoculación intratorácica. CxFV infectó a *Cx. pipiens* después de inoculaciones intratorácicas, pero no a través de la exposición oral. Posteriormente, los

ensayos de competencia vectorial frente a RVFV se realizaron en co-infección con CxFV y un flavivirus de mosquito de circulación natural, respectivamente, en *Cx. pipiens* y *Ae. vexans*. Ambas poblaciones locales mostraron ser competentes para RVFV después de exposición oral. Por otra parte, CxFV ni RVFV interfirieron con su respectiva infección, mientras que el flavivirus de mosquito de circulación natural, aunque no evitó la transmisión, moduló la susceptibilidad de infección con RVFV en *Ae. vexans*, sugiriendo su potencial uso como agente biológico para la prevención de la transmisión de RVFV.

Finalmente, para evaluar nuevas alternativas para la detección de virus circulantes y vigilancia de arbovirus (Capítulo 3), tarjetas FTA con cebo de miel fueron expuestas a mosquitos capturados en el campo durante jornadas de vigilancia entomológica para su posterior análisis mediante secuenciación de nueva generación. Virus asociados a artrópodos y plantas fueron identificados en las FTAs y genomas virales casi completos fueron obtenidos, lo que sugiere una buena preservación de ARN viral. Para confirmar la presencia de virus asociados con mosquitos en los especímenes capturados, los pools de mosquitos fueron analizados usando PCR de transcripción reversa y primers especie-específicos diseñados a partir de las secuencias obtenidas de las FTAs. Virus relacionados con *Alphamesonivirus, Quaranjavirus* y *Bunyavirales* no clasificados fueron detectados en mosquitos de Cataluña constituyendo el primer registro de distribución de estos virus en mosquitos europeos. La detección de ISVs en la saliva de mosquitos de forma silvestre demuestra la viabilidad de este método para monitorear la fracción transmisible del virroma de mosquitos y su utilidad en la vigilancia de arbovitus.

El presente trabajo contribuye con información para un mejor entendimiento de los factores detrás de la estructura del microbioma de mosquitos locales y su potencial influencia en la competencia vectorial frente a arbovirus. Adicionalmente, provee un nuevo método para complementar la vigilancia de arbovirus en zonas susceptibles, así como, para la detección de nuevos virus circulantes y potencialmente patogénicos.

RESUM

A Europa, l'amenaça del ressorgiment i introducció de malalties transmeses per mosquits, com la malària i la febre de la vall del Rift, ha despertat un nou interès en l'estudi del microbioma associat amb mosquits autòctons per a una millor comprensió de les interaccions mosquit–patogen amb la finalitat de desenvolupar eines de vigilància vectorial i control ecològicament més adequades - En conseqüència, la tesi present es va enfocar en i) explorar la microbiota de l'*Anopheles atroparvus*, ii) avaluar la influencia de flavivirus insecto-específic en la competència vectorial del *Culex pipiens* i de l'*Aedes vexans* d' Europa per a la transmissió de RVFV, i iii) Aplicar metagenòmica a targetes FTA com a nou mètode per a la detecció de virus i vigilància d'arbovirus.

Al primer capítol, es va establir una colònia de laboratori d' *An. atroparvus* del Delta de l' Ebre i es va actualitzar el seu protocol de cria. El seqüènciament del gen bacterià 16S rRNA va mostrar que l'ambient de cria, fisiologia i hàbits de farratge va influir en la microbiota tant de mosquits de camp com de laboratori. Addicionalment, els anàlisis de diversitat van mostrar variació entre els diferents estadis de desenvolupament silvestres i un declivi de la diversitat en el grup de femelles de la desena generació de laboratori F10, tot i això, van conservar una fracció significativa de la microbiota de les femelles silvestres. Finalment, proteobactèries Gram-negatives van predominar en la microbiota de l' *An. atroparvus,* entre les quals *Pseudomonas*, *Asaia* i *Serratia* van ser identificades com a candidates potencials per al control vectorial local.

Per avaluar la influencia de ISFVs en la competència vectorial enfront de la transmissió de RVFV (Capítol 2), primer es va estudiar la infecció de Culex flavivirus (CxFV) a *Cx. pipiens* a través d'exposició oral i inoculació

intratoràcica. CxFV va infectar a *Cx. pipiens* després d'inoculacions intratoràciques però no a través de l'exposició oral. Posteriorment, els assajos de competència vectorial enfront de RVFV es van realitzar en co-infecció amb CxFV i un flavivirus de mosquit de circulació natural, respectivament, en *Cx. pipiens* i *Ae. vexans*. Ambdues poblacions locals van mostrar ser competents per a RVFV després d'exposició oral. Per altra banda, ni CxFV ni RVFV van interferir en la seva respectiva infecció, mentre que el flavivirus de mosquit de circulació natural, encara que no va a aturar la transmissió, va modular la susceptibilitat d'infecció amb RVFV en *Ae. vexans*, suggerint el seu potencial ús com agent biològic per a la prevenció de la transmissió de RVFV.

Finalment, per a avaluar noves alternatives per a la detecció de virus circulants i vigilància d'arbovirus (Capítol 3), targetes FTA amb esquer de mel van ser exposades a mosquits capturats en el camp durant jornades de vigilància entomològica per a la seva posterior anàlisi mitjançant seqüenciació de nova generació. Virus associats a artròpodes i plantes van ser identificats en les FTAs i es van obtenir genomes virals gairebé complets, el que suggereix una bona preservació d' ARN viral. Per a confirmar la presència de virus associats amb mosquits en els espècimens capturats, els pools de mosquits van ser analitzats utilitzant PCR de transcripció reversa i primers espècie-específics dissenyats a partir de les seqüències obtingudes de les FTAs. Virus relacionats amb *Alphamesonivirus, Quaranjavirus* i *Bunyavirales* no classificats van ser detectats en mosquits de Catalunya constituent el primer registre de distribució d'aquests virus en mosquits europeus. La detecció d' ISVs a la saliva de mosquits de forma silvestre demostra la viabilitat d'aquest mètode per a fer monitoreig de la fracció transmissible del viroma de mosquits i a seva utilitat en la vigilància d'arbovirus

INTRODUCTION

Microorganisms are a highly diverse and ubiquitous group that has populated the Earth for over 3.5 billion years (Schopf, 1993). Over evolutionary time, microorganisms have played essential roles in the evolution and functioning of the ecosystems and other living organisms (McFall-Ngai et al., 2013). For example, the biosphere, as we know it, is product of the metabolism of ancestral microorganisms and their interactions. While, terrestrial and marine microbes were (and still are) the main drivers of global nutrient cycles (e.g., nitrogen and carbon) (Blaser et al., 2016), early photosynthetic microorganisms (i.e., cyanobacteria) liberated great amounts of molecular oxygen into the atmosphere favoring gene and species diversification (David and Alm, 2010). Moreover, strong evidence supports the evolution of multicellular eukaryotes (i.e., plants and animals) from the long-term association – **symbiosis** - of unicellular organisms (i.e., bacteria or archaea), their further combination and diversification (reviewed in Archibald, 2015).

Following the development of microscopy and with the advent of culture-independent technologies (**Box 1**), microbial symbiotic relationships have also garnered relevance shaping the evolution of higher organisms. It is clear that most, if not all, metazoans – **the hosts** – are colonized by a dynamic assemblage of microorganisms – **the symbionts** – with whom they have established complex symbiotic networks (Gilbert et al., 2012). Archaea, bacteria, algae, fungi, and protozoa, which may comprise the cellular component of this consortium – **the microbiota** –, interact with one another, as well as with internal and external structural elements (i.e., lipids, proteins and polysaccharides), mobile genetic elements (i.e., **viruses**, phages and plasmids), and metabolites (i.e., signaling molecules, toxins, organic and inorganic molecules) produced by both, the microbiota and the host under the influence of their surrounding habitat. Currently, this entire interacting conglomerate is known as **the microbiome** (Berg et al., 2020).

Box 1: 16S rRNA sequencing and Shut-gun metagenomics for bacterial and virus detection

Next generation sequencing approaches, such as **16S rRNA gene sequencing** and shutgun metagenomics have been widely used to characterize the composition and functional capacities of the microbiome of biological and environmental samples.

Ribosomal RNA (rRNA) is non-coding RNA that predominates in all cells. In prokaryotes, it is organized in two subunits: large (LSU), containing 23S and 5S rRNA molecules, and small (SSU) formed by a single rRNA molecule, 16S (Woese, 1987). All three genes (23S, 5S and 16S) form a gene cluster linked by internal transcribed spacer (ITS) regions. Among these, due to the presence of conserved and variable polymorphic regions (V1 – V9), the 16S rRNA gene has been widely used as a molecular marker for the taxonomic characterization of bacterial diversity, as well as, inferring phylogenetic relationships between bacterial taxa (Woese, 1987; Palys, Nakamura and Cohan, 1997; Kolbert and Persing, 1999). The extended use of the bacterial 16S rRNA gene has provided a large sequence database of over 90.000 nucleotide sequences to compare with and identify unknown strains (Clarridge, 2004).

Shotgun metagenomics is an untargeted sequencing that enables a much deeper characterization of the genetic diversity present in a sample. Besides taxonomic and functional information from the sequenced genomes, a larger number of species per sample can be obtained when compared to 16S rDNA amplicon sequencing; unculturable bacteria and viruses can be identified (Sharpton, 2014; Laudadio et al., 2018).

The structure (i.e., diversity and composition) of the microbiome is highly variable and primarily shaped by the environment. Since a significant fraction of the microbiome's diversity is acquired from the habitat (natural or artificial) where the host develops, microbial communities harbored by organisms that live in the same environment present higher similarities than those of allopatric hosts (Yatsunenko et al., 2012; Park et al., 2019). Likewise, throughout the host's lifecycle, symbiont communities can be distinguished between early and mature stages (Yatsunenko et al., 2012) and between sex/genders (male/female) (Ding and Schloss, 2014; Chen et al., 2016). In addition, the location within the host (e.g., skin/cuticle or gut/midgut) may also influence the structure of the microbiome (Ding and Schloss, 2014; Park et al., 2019) due to variation in local abiotic (i.e., physiochemical factors and barriers) and biotic (i.e.,

presence/absence of other symbionts) factors (Zilber-Rosenberg and Rosenberg, 2008; Theis et al., 2016). Besides horizontal diversity acquisition, a subset of the microbiome can be maternally transferred and passed over generations (Ferretti et al., 2018; Kowallic and Mikheyev, 2021). For this reason, hosts that are phylogenetically related (e.g., clades/species) tend to exhibit more similar microbiomes than those harbored by distant or unrelated host groups (e.g., mammals and invertebrates) (Jones, Gonzales-Sanchez and Fierer, 2013; Brooks et al., 2016).

Symbiotic relationships within the microbiome and, between the microbiome and its host, may produce multiple interactions with a wide range of effects. Primarily, these interactions may vary from i) advantageous, when at least one of the interacting partners is beneficiated (e.g., commensalism, mutualism); to ii) neutral, when no effect is observed on either partner; or to iii) adverse, when one or more of the interacting partners is harmed or eliminated (e.g., parasitism, predation, competition) (Berg et al., 2020). Shifts on microbe-microbe and/or microbe-host interactions can be circumstantial and produced, among others, by environmental variation and/or alterations on the diversity and composition of the microbiome (Theis et al., 2016). As a result, some symbionts and/or their effect could be transient, persist over a limited period of time (e.g., season or life stage), or be permanent. Symbionts that maintain advantageous or neutral effects on the host are mostly inherited, preserved over time and become part of the native microbiome of the population (Zilber-Rosenberg and Rosenberg, 2008).

Despite adverse symbionts have been the most extensively studied due to their direct repercussions on human, animal and plant health, in recent years, advantageous symbionts have gained considerable attention. They have been associated with relevant biological traits of the host such as development, nutrition, reproduction, immunity, and even behavior (Dillon and Dillon, 2004; Ezenwa et al., 2012; Hooper, Littman and Macpherson, 2012; Eleftherianos et al., 2013; Engel and Moran, 2013; Brune, 2014). It has also been established that symbionts

may aid the host to better adapt to a changing environment by microbial gene amplification and later, modifying its phenotype through gene transfer (Rosenberg et al., 2010). Consequently, due to these strong interdependencies between hosts and their associated microbiome, they are now considered as a composite that live, develop and evolve together as a unit of selection – **the holobiont** (Zilber-Rosenberg and Rosenberg, 2008).

Over the past decades, the concept of the holobiont has raised new interests in vector biology since several phenotypes in vector populations are strongly influenced by their microbiome, and alterations in some of these traits may influence (negatively or positively) their ability to transmit infectious agents – **their vectorial capacity**. Entomologically, the vectorial capacity describes disease transmission intensity as the expected number of infective bites that would be originated, on a single day, from all the mosquitoes biting a fully infectious individual introduced into a susceptible population (Garret-Jones, 1964). In its equation (Macdonald. 1956), vectorial capacity incorporates the period of time required by the pathogen to complete its cycle within the vector (from infection to infectious) – the extrinsic incubation period (N), and, key bionomic parameters of the vector mosquitoes such as population density (*m*), biting rate (*a*), the probability of daily survival (*p*), and the ability to acquire, maintain and transmit the pathogen – **the vector competence** (*b*) (Garret-Jones, 1964). Each of these variables being sensitive to symbiotic changes.

$$Vectorial\ Capacity = ma^2bp^N/-log_e p$$

Firstly, (**N**) the extrinsic incubation period (EIP) establishes the number of infected mosquitoes with a lifespan long enough to transmit a pathogen. Shorter EIPs may increase the number of infectious individuals and therefore the transmission risk, while longer EIPs may produce the opposite effect. In

Aedes aegypti for example, a previous infection with the intracellular bacterium *Wolbachia* (*w*Mel) yielded a significant delay in the time it took for dengue virus (DENV-3) to disseminate into the saliva and become infectious. Extending the EIP for DENV implied a reduction in the number of infectious mosquitoes and consequently the reduction in the transmission potential (Ye et al., 2015).

Secondly, (***m***) mosquito population density refers to the number of vector mosquitoes in proportion to host. The higher the mosquito density, the greater the transmission risk. Interfering with larval development would severely reduce adult population sizes, for example, first-instar larvae of *Ae. aegypti* and *Anopheles gambiae* were unable to molt in the absence of gut microbiota (Coon et al., 2014; Valzania et al., 2018), and when *Asaia* and *Acinetobacter* were eliminated or reduced in *Anopheles stephensi* and *Ae. aegypti,* respectively, developmental times were extended (Chouaia et al., 2012; Martinson and Strand, 2021). Likewise, interfering mosquito's fecundity, which translates in decreased reproductive outcomes, may also affect population densities. In *Aedes, Anopheles* and *Culex* mosquitoes *Wolbachia* infections may produce cytoplasmic incompatibility (Yeap et al., 2011; Sicard, Bonneau and Weill, 2019; Ant et al., 2020; Adams et al., 2022) and the Gram-negative bacterium *Chromobacterium* may reduce the number of eggs laid, their viability and hatching rate in *Anopheles coluzzii* (Gnambani et al., 2020). Additionally, siderophores, which are microbial (e.g., *Serratia* sp., *Enterobacter* sp.) metabolites that now can be considered part of the microbiome, have also seen to decrease population's fecundity in *An. gambiae* (Ganley et al., 2020). The most common approach to target mosquito abundance is the use of insecticides. *Bacillus thuringiensis israelensis* (Bti) is the most environmentally-safe bioagent that is being widely used for its larvicidal effects on nuisance dipterans (Brühl et al., 2020) and now it is being tested as a toxic sugar bait for adult mosquitoes (Davis et al., 2021). Currently, entomopathogenic fungi (e.g., *Metarhizium* and *Beauveria*), due to highest mortalities and wide spectrum

(Blanford et al., 2012; Wei et al., 2017), and other bacteria are being considered as potential insecticide alternatives. *Chromobacterium*, for example, presented insecticidal effect against both, immature and adult stages in several mosquito species (Ramirez et al., 2014; Short et al., 2018; Ganmbani et al., 2020), and in *An. gambiae* caused midgut transcriptional modifications similar to those of insecticide exposure and produced higher mortalities in subsequent generations (Short et al., 2018). However, there is increasing evidence of the correlation between the microbiota and insecticide resistance. Most studies report differences in symbiont communities between susceptible and resistant populations and identify bacterial taxa that may confer xenobiotic tolerance (Dada et al., 2018; Arevalo-Cortez et al., 2020; Omoke et al., 2021; Wang et al., 2021). For instance, when resistant *Anopheles albimanus* were exposed to organophosphates (OPs) showed a reduced microbial diversity where bacteria with xenobiotics-degrading functions were selected (Dada et al., 2018). Reducing mosquito densities would significantly reduce vector-host contact and, therefore, the transmission risk.

Thirdly, (***a***) biting rate or blood feeding frequency represents the probability of a vector mosquito to feed on a host on a single day. Host-seeking behavior and blood-feeding propensity are responsible for biting frequencies. Infections with the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* have shown to suppress host-seeking behavior, as well as, reduce blood feeding propensity in *Aedes*, *Anopheles* and *Culex* mosquitoes (Blanford et al., 2005; Scholte, Knols and Takken 2006; Howard et al., 2010). Similarly, in *Ae. aegypti*, in addition to *Serratia* (Koslova et al., 2021), siderophores (Ganley et al., 2020) and neuropeptides suppressed host-seeking behavior and prevented biting (Duvall et al., 2019). Since vector species with increased willingness to blood-feed rise the possibilities of infection and pathogen transmission, modulation of host-seeking and/or blood-feeding behavior may substantially reduce transmission risk.

Fourthly, (***p***) the probability of mosquito daily survival may primarily depend on environmental conditions. However, through the introduction of life-shortening microorganisms mosquito's longevity could be modulated, as it is the case of *Wolbachia*, entomopathogenic fungi (e.g., *Metarhizium* and *Beauveria*), and densovirus (Carlson, Suchman and Buchatsky, 2006; McMeniman et al., 2009; Mnyone et al., 2011). Since older individuals are more likely to be infectious, shortening mosquito lifespan may not only reduce vector-host contact, but also interrupt pathogen's EIPs and consequently, the number of infectious individuals (Cook, McMeniman and O'Neill, 2008).

Finally, (***b***) vector competence represents the proportion of mosquitoes that, after an infectious blood meal, are capable of subsequently transmitting a pathogen by bite (Hardy et al. 1983). For an effective transmission, pathogens need to overcome a series of barriers before disseminating into the saliva (**Figure 1**). Following ingestion, pathogens reach the midgut where they face internal physio-chemical and mechanical obstacles (e.g., digestive enzymes, peritrophic matrix, and the epithelium), mosquito innate immune responses (i.e., RNAi) and interact with the microbiome – **the midgut infection barrier (MIB)**. Once they have overcome the MIB, cross the midgut epithelium – **midgut escape barrier (MEB)** – enter the haemocoel from where they may disseminate to secondary organs (e.g., fat body, muscles, nerves and salivary glands) **(Figure 1C)**. At last, pathogens need to infect and breach the salivary glands – **salivary gland infection/escape barrier (SGIB/SGEB)** – to be released to the saliva for their inoculation into a new host during subsequent blood meals (Kramer and Ciota, 2015). It is known that the microbiome in key mosquito organs, such as the midgut, may influence vector competence (Tchioffo et al., 2016). Midgut microbiota, as part of the MIB, plays a significant role in mosquito's infection susceptibility. Bacteria such as *Chromobacterium*, *Proteus* and *Paenibacillus* have seen to significantly increase *Ae. aegypti* resistance to dengue virus (DENV-2) (Ramirez et al., 2012; 2014), while *Serratia* increased permissiveness to DENV infection (Wu et al., 2019) and enhanced

DENV and CHIKV replication (Apte-Deshpande et al., 2012; 2014). Similarly, transient trypanosomes in *Anopheles* mosquitoes increased their susceptibility to *Plasmodium* parasites (Dieme et al., 2020), whereas, enterobacteria (e.g., *Enterobacter* and *Serratia*) inhibited *Plasmodium* development (Gonzalez-Ceron et al., 2003; Bando et al., 2013; Dennison et a., 2016). In addition to pathogen-microbiota interactions, **virus-virus interactions** have also seen to affect vector competence through synergistic or antagonistic effects, facilitating or interfering the replication/transmission of the other virus, respectively (Muturi, Buckner and Bara, 2017). It is increasingly known that mosquito viral metagenome – **the virome** – is more diverse than previously thought, mostly composed by RNA viruses, among which, arthropod-borne viruses – **arboviruses** – and **insect-specific viruses** (ISVs) (**Box 2**) can be distinguished. In one hand, arboviruses are pathogenic dual-host viruses that replicate in, both, vertebrate and invertebrate cells; and on the other hand, the recently described and continuously reported ISVs, which are host-restricted to replication in invertebrate cells and may not seem to infect vertebrates (Bolling et al., 2015). *In vitro* and *in vivo* co-infection (simultaneously) and sequential infection (i.e., subsequent infection with a second virus) assays have shown variable outcomes from arbovirus-arbovirus and ISV-arbovirus interactions. *Aedes* cell lines persistently infected with ISVs cell-fusing agent virus (CFAV) and Phasi Charoen-like virus (PCLV) limited ZIKV and DENV replication and inhibited La Crosse virus (LACV) growth (Schultz, Frydman and Connor, 2018). A recent work that performed co-infections with DENV and Zika virus (ZIKV) (family *Flaviviridae*) in *Ae. aegypti* yielded higher ZIKV infection and dissemination rates with a higher number of cDNA copies, and reported that ZIKV-DENV interaction favored ZIKV transmission. In the same study, while ZIKV in mono-infections presented a higher number of cDNA copies than in co-infection, DENV cDNA levels were higher in co- than in mono-infections (Chaves et al., 2018). Moreover, a positive correlation for CHIKV infection was observed in *Aedes koreicus* in co-infection with the novel naturally circulating

ISV soberno-like virus, Wiesbaden virus (WBDV) (Jansen et al., 2021). Furthermore, ISVs intrathoracic inoculations in *Culex* mosquitoes interfered/suppressed Japanese encephalitis virus (JEV) and West Nile virus (WNV) replication (Sudeep et al., 2015; Colmant et al., 2018). Among vectorial capacity parameters, vector competence allows inferring the infection susceptibility and transmission potential of a mosquito population, and, owing the difficulty of an appropriate estimation of some of the other bionomic parameters (e.g., biting frequencies and survivor) (Lounibos and Kramer, 2016), the assessment of the vector competence of local mosquitoes is crucial to determine whether they pose a threat of an epidemic transmission or pathogen emergence in a given region.

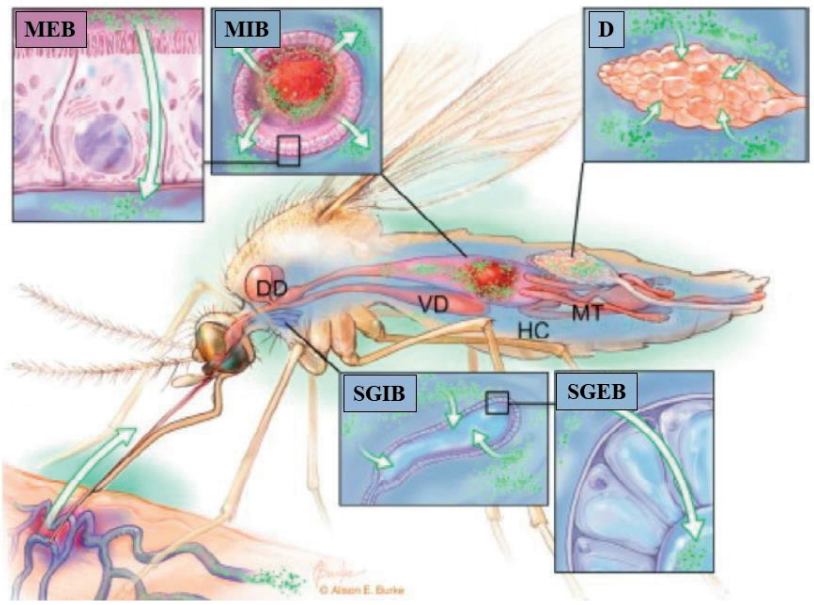

Figure 1. Anatomical and physiological barriers that condition mosquito vector competence. **MIB**, midgut infection barrier; **MEB**, midgut escape barrier; **D**, dissemination to secondary organs; **SGIB**, salivary gland infection barrier; **SGEB**, salivary gland escape barrier. DD, dorsal diverticulum; VD, ventral diverticulum; HC, haemocoel; MT, Malpighian tubules. (Figure adapted from: Chamberlain and Sudia, 1961).

Box 2: Insect-specific viruses: Discovery, classification and transmission

Insect-specific viruses (ISVs) are mostly RNA viruses with a worldwide geographic distribution that are common in natural insect populations. They were first described with the isolation of cell-fusing agent virus (CFAV) from an *Aedes aegypti* cell line (Stollar and Thomas, 1975). Years later, CFAV-like viruses Kamiti River virus (KRV), *Culex* flavivirus (CxFV) and *Aedes* flavivirus (AeFV) were isolated, respectively, from field-collected immature (larvae and pupae) *Aedes macintoshi* from Kenya (Crabtree et al., 2003), *Culex* and *Aedes* mosquitoes from Japan (Hoshino et al., 2007; 2009). Since then, with the advances in virus detection (e,g., metagenomics and full genome sequencing) and an up-scale in arboviral surveillance, there has been a significant increase in the discovery and isolation of ISVs.

Novel ISVs have been classified within plant and animal infecting families *Birnaviridae*, *Bunyaviridae*, *Flaviviridae*, *Mesoniviridae*, *Nodaviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae*, *Tymoviridae*, to name a few (Bolling et al., 2015; Atoni et al., 2019). The largest number of discovered ISVs corresponds to *Flaviviridae* (genus *Flavivirus*) (Bolling et al., 2015). Since in the present thesis the role of two insect-specific flaviviruses (ISFs) in arbovirus transmission is studied a brief description of their structure is provided. As all flaviviruses, ISFs possess a single-stranded positive-sense genome that encodes a single open reading frame (ORF), which in turn encodes a large polypeptide that is co- and post-transcriptionally sliced by host and viral enzymes into ten proteins: three structural proteins, envelop (E), membrane/pre-membrane (M/prM) and capsid (C); and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The later, involved in viral replication and assembly, and modulation of host responses (Fernandez-Garcia et al., 2009; Blitvich and Firth, 2015).

Phylogenetically, ISFs can be divided into two groups, single-host (or classical ISFs) and dual-host ISFs. The former, cluster separately from all known dual-host viruses, while the later, is related to known arboviruses, although, there is no evidence of vertebrate cells infection (Blitvich and Firth, 2015). Among classical ISFs, CFAV, AeFV and CxFV are being continuously detected in field-caught mosquitoes all over the globe (Crabtree et al., 2009; Hoshino et al., 2009; Roiz et al., 2012; Jeffries et al., 2020; Martin et al., 2020; Guarido et al., 2021).

ISVs transmission cycle is still unknown. Since ISVs (mainly flaviviruses) have been found infecting field immature stages and males (Saiyasombat et al., 2011; Haddow et al., 2013;) and transovarial transmission has been evidenced from field-caught mosquitoes (Saiyasombat et al., 2011) it is thought that vertical transmission is the main route of ISVs maintenance in nature. Vertical, horizontal and venereal transmission have been proven experimentally (Lutomiah et al., 2007; Logan et al., 2022).

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Phylogenetic analyses have shown that ISVs may represent ancestral lineages of dual-host viruses and suggested that arboviruses originated from ISVs (Cook and Holmes, 2006; Marklewitz et al., 2015; Walker et al., 2015). This hypothesis may imply the evolution of ISVs into new emerging and potentially pathogenic viruses. Consequently, there is a growing interest in their study and surveillance.

In the current epidemiological setting, mosquito-borne diseases (MBD) are still a major veterinary and public health concern, and despite global efforts they are in continuous expansion. The latest World Health Organization (WHO) malaria report estimated 241 million cases of malaria, with more than 600.000 associated deaths, about 14 million more cases and nearly 70.000 more deaths in 2020 than in 2019 (WHO, 2021). It has been established that the SARS-CoV-2 pandemic in 2020 exacerbated malaria incidence due to deprived health services (Commonwealth, 2022), situation that could be extrapolated to other MBD. To date, only in the Americas, nearly three million cases of arboviral diseases have been reported, among which, dengue accounts for the vast majority (2.5 million cases) followed by chikungunya and zika with 250.000 and 31.000 cases respectively (PAHO, 2022).

Globalization, climate change and human activities play a significant role in the epidemiology of MBD. The increase of international travel, migration and commerce trends from/with endemic countries have expanded pathogen's and vector's geographic range. While, climate change has modified the temperature and rain/drought cycles extending transmission seasons, shortening vector developmental cycles and pathogen's EIPs, and, creating favorable conditions for vector establishment in newer areas (Mayers, Tesh and Vasilakis, 2017; Musso et al., 2018). As a consequence, in the last decade, the number of imported cases of malaria have increased in Europe (Piperaki and Daikos, 2010), and sporadic episodes of autochthonous transmission of dengue,

Zika, chikungunya, and West Nile viruses have been reported (Angelini et al., 2007; La Ruche et al., 2010; Brady and Hay, 2019; Franke, 2019; Garcia San Miguel Rodriguez-Alarcon et al., 2020; Barzon et al., 2021). The likelihood that autochthonous mosquito populations might be involved in *Plasmodium* parasites and arboviruses transmission poses on alert for the resurgence of malaria and the introduction of other sanitary relevant arboviral diseases into the continent.

Mosquitoes from the *Anopheles maculipennis* subgroup are considered the dominant vector species of malaria parasites in Europe (Sinka et al., 2010), among which *Anopheles atroparvus* is the most abundant and widely distributed (Hertig et al., 2019). Formerly, *An. atroparvus* was implicated in the transmission of local strains of both *Plasmodium vivax* (Bueno-Mari and Jiménez-Peydró, 2012) and *Plasmodiun falciparum* (Jetten and Takken, 1994). Currently, to estimate the risk of malaria re-introduction it is critical to assess the competence of local anopheline populations for the transmission of the most commonly imported *Plasmodium* parasites. Since laboratory breeding may alter the microbial diversity of field-colonized vector mosquitoes (Rani et al., 2009; Duguma et al., 2015; Dada et al., 2020), and consequently their vector competence (Boissiere et al., 2012; Osei-Poku et al., 2012) it is crucial to have access to an updated *An. atroparvus* laboratory colony (Chapter 1, assay 1). Moreover, a comprehensive characterization of its bacterial communities from the field and during the laboratory colonization process is essential for a better interpretation of vector competence outcomes and for the design of more accurate local prevention and control strategies (Chapter 1, assay 2).

Aedes and *Culex* species are considered the primary vectors of arboviruses. In Europe, the potential role of native and invasive populations in arbovirus transmission has been proven experimentally (Vega-Rua et al., 2013; Blagrove et al., 2016; Brustolin et al., 2016; Ciocchetta et al., 2018; Mariconti et al., 2019; Nuñez et al., 2020), and arbovirus infections have been identified in local mosquitoes (Hesson et al., 2015; Patsoula et al., 2016; Aranda et al., 2018). Besides

arbovirus circulation, ISVs have also been identified in European mosquitoes (Calzolari et al., 2012). In this context, Rift Valley fever (RVF), another zoonotic arboviral disease, threatens with its introduction into the European continent. Due to its epidemic potential and lack of countermeasures RVF belongs to the World Organization for Animal Health (OIE)'s list of notifiable animal diseases of concern and it is prioritized for WHO research and development in public health emergency contexts. Despite no cases of RVF have been detected in Europe so far, the Spanish Mediterranean showed a high suitability for RVF occurrence (Sanchaz-Viscaino et al., 2013). Since *Aedes* and *Culex* mosquitoes have been incriminated as primary vectors of its causative agent, Rift Valley fever phlebovirus (RVFV) (**Box 3**), in endemic areas (Abdo-Salem et al., 2012), the competence of Catalonian populations of *Culex pipiens* (**Box 4**) and *Aedes albopictus* has been proven experimentally (Brustolin et al., 2017). However, the infection susceptibility and transmission potential of wild populations of *Aedes vexans* (**Box 4**), which is considered one of the primary vectors of RVFV in Africa (Ndiaye et al., 2016; Talla et al., 2016; Sang et al., 2017) and widely distributed in Europe, have not been assessed yet (Chapter 2, assay 2). In addition, it is relevant to evaluate arbovirus-ISVs interactions between locally circulating ISVs and potentially introduced arboviruses for a better understanding of their dynamics and role in arbovirus transmission of native mosquito populations. Herein, *Cx. pipiens* and *Ae. vexans* from Catalonia were challenged for RVFV transmission in co-infections with ISVs of local circulation (i.e., *Culex* flavivirus and mosquito flavivirus) (Chapter 2, assays 1 and 2).

Box 3: Rift Valley fever phlebovirus: Structure, transmission, and epidemiology

Rift Valley fever phlebovirus (RVFV) is an enveloped negative single-stranded RNA virus with a genome divided into three segments designated small (S), medium (M) and large (L). The S segment, of ambisense polarity, encodes (N) and the nonstructural protein NSs, which comprises the main factor of virulence. The M and L segments, of negative sense, encode respectively for glycoproteins and the non-structural protein Nsm, and the RNA-dependent RNA polymerase. All three ribonucleocapsids (S, M, and L) are surrounded by the viral envelop, which is a lipidic bilayer covered by capsomers formed by glycoproteins, Gc and Gn heterodimers (Mansfield et al., 2015; Pepin et al.,2010; Ikegami, 2012). There are 15 RVFV linages with limited genetic diversity and all strains are closely related at amino acid and nucleotide levels (Ikegami, 2012).

RVFV is transmitted horizontally between animals (e.g., goats, sheep, camels) and from sick animals/humans to humans through direct contact with infected body fluids, meat or carcasses or by infected mosquito bites (Gibson et al., 2022). In vector mosquitoes, RVFV is suggested that is transmitted vertically from infected floodwater *Aedes* females to their eggs where it is preserved during dry periods, while *Culex* may act as amplifying vectors (Bird et al., 2009; Pepin et al., 2010). Wild and domesticated ruminants are mainly affected by the virus presenting a mild-to-severe febrile illness with high mortalities in newborns and abortion in pregnant animals. Less than 1% of human sporadic infections develop blindness, encephalitis, hemorrhagic fever and death (Chevalier et al., 2010; Mansfield et al., 2015).

Since its first detection in Kenya in 1931, RVFV has caused several epidemic and epizootic outbreaks through the Sub-Saharan Africa and Egypt, and has spread outside continental Africa to Madagascar, the Comoro and Mayotte islands, Saudi Arabia and Yemen. There has been a constant circulation of the virus and yearly, at least one outbreak is produced, the latest being reported in Mauritania between August and October 2022 (WHO, 2022). Rift Valley fever outbreaks are highly associated to heavy rain and flooding seasons (e.g., El Niño Southern Oscilation - ENSO), which coincide with highest mosquito proliferation (Paweska, 2015).

Box 4: *Culex pipiens* and *Aedes vexans* mosquitoes

Culex pipiens, the "common-house mosquito", is ubiquitous in temperate regions. It is a species complex that occurs in two biological forms or biotypes, *pipiens* and *molestus*. Morphologically, these biotypes are no differentiable, but present genetic, physiological and behavioral differences (Vinogradova, 2003). *Culex pipiens pipiens*, which is anautogenous, eurygamous and heterodynamic, which means that require a blood-meal for the first egg-lay, mate in open spaces and undergoes diapause, respectively. Inhabits above-ground and have preference for feeding on birds. Whereas *Culex pipiens molestus*, is autogenous, stenogamous and homodynamic, live underground and mammals are its host of preference. In the Mediterranean basin both biotypes are sympatric and frequently hybridize (Gomes et al., 2010; Amraoui et al. 2012). *Culex pipiens* hybrid, present both feeding-behavioral patterns biting birds and mammals.The *Culex pipiens* complex has been incriminated in the transmission of arboviruses such as WNV, USUV and SINDV (Amraoui et al. 2012; Brugman et al. 2018).

Aedes vexans is a floodwater mosquito widely distributed in rural areas of the Holarctic (Becker et al., 2003). It is heterodynamic (undergoes diapause), its eggs hatch massively after flooding episodes and complete its cycle in few days (Miller et al., 2002). This species present low host preference among mammals and humans. It has been incriminated in the transmission of several arboviruses (e.g., WNV, Tahyna virus (TAHV), Batai virus (BATV), RVFV) (Gligić and Adamović, 1976; Anderson et al., 2015; Talla et al., 2016; Scheuch et al., 2018).

In this scenario, and since human incursion to sylvatic areas and changes in the land use (e.g., deforestation, agriculture, husbandry and urbanization) have favored the contact of vectors with new pathogens and contributed to their dispersal (Mayers, Tesh and Vasilakis, 2017; Musso et al., 2018), it is essential to implement new approaches in routine entomological surveillance that allow to increase the knowledge of the viral diversity harbored by vector mosquitoes and therefore can be used as early indicators for local transmission and outbreaks of known and unknown circulating viruses. The present work proposes a novel approach using next generation sequencing applied on honey-baited filter paper cards used in entomological surveys to detect the transmissible fraction of the mosquito's virome (Chapter 3).

Although, significant improvements in chemotherapy, vector control and surveillance have been achieved in the last years, the harmful ecological effects of insecticides and the appearance of insecticide-resistant mosquitoes have raised the interest to find novel and more ecologically adequate alternatives to prevent MBD. The study of the microbiome (herein, bacteria and viruses) of vector mosquitoes may allow a better understanding of microbe-mosquito-pathogen interactions (one of the intrinsic factors behind their infection susceptibility and vector competence) and may help harnessing microbial constituents to affect the physiology of local mosquitoes, which is critical for the development of novel and more effective prevention and control strategies.

OBJECTIVES

The primary objective of the current thesis is to further our understanding of the role of microbiota-virome in vector competence and its potential for surveillance and control of pathogens transmitted by European vector mosquitoes. In order to achieve this main goal, the current thesis is divided into three chapters with the following specific objectives:

Chapter 1: Microbiota Profiling of Sylvan and Laboratory *An. atroparvus*

i) Establishing a new field-colonized standard laboratory colony of *Anopheles atroparvus* from the Ebro Delta, a former malaria endemic area of Spain, for malaria research purposes.

ii) Comprehensively profiling the bacterial community composition of an autochthonous population of *An. atroparvus* and determining how this varies throughout the mosquito lifecycle and laboratory colonization process.

Chapter 2: Microbiome and Vector Competence: Influence of ISFs on RVFV Transmission

i) Assessing the influence of insect-specific flaviviruses (ISFs) on the vector competence of Catalonian *Culex pipiens* and *Aedes vexans* for the transmission of RVFV to understand ISFs dynamics and their role in their mosquito hosts as potential control tool.

ii) Assessing the vector competence of field-captured *Aedes vexans* from Catalonia for Rift Valley fever phlebovirus (RVFV) to estimate the transmission risk and design more adequate vector control and disease prevention strategies.

Chapter 3: Metagenomics: New Insights for Virus Detection

iii) Applying metagenomics on honey-baited filter paper cards (FTA) used in entomological surveys as a new approach for the detection of the transmissible fraction of the mosquito's virome and for arbovirus surveillance.

(Fragment, original illustration: Abraham Birnberg, 2021)

CHAPTER 1

Microbiota Profiling of Sylvan and Laboratory *Anopheles atroparvus*

ASSAY 1

Laboratory colonization and maintenance of *Anopheles atroparvus* from the Ebro Delta, Spain

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ABSTRACT

Historically, *Anopheles atroparvus* has been considered one of the most important malaria vectors in Europe. Since malaria was eradicated from the European continent, the interest in studying its vectors reduced significantly. Currently, to better assess the potential risk of malaria resurgence on the continent, there is a growing need to update the data on susceptibility of indigenous *Anopheles* populations to imported *Plasmodium* species. In order to do this, as a first step, an adequate laboratory colony of *An. atroparvus* is needed.

Anopheles atroparvus mosquitoes were captured in rice fields from the Ebro Delta (Spain). Field-caught specimens were maintained in the laboratory under simulated field-summer conditions. Adult females were artificially blood-fed on fresh whole rabbit blood for oviposition. First- to fourth-instar larvae were fed on pulverized fish and turtle food. Adults were maintained with a 10% sucrose solution *ad libitum*.

An *An. atroparvus* population from the Ebro Delta was successfully established in the laboratory. During the colonization process, feeding and hatching rates increased, while a reduction in larval mortality rate was observed.

The present study provides a detailed rearing and maintenance protocol for *An. atroparvus* and a publicly available reference mosquito strain within the INFRAVEC2 project for further research studies involving vector parasite interactions.

Keywords: *Anopheles atroparvus*, Colonization, Malaria, Europe.

BACKGROUND

In Europe and the Middle East, dominant *Anopheles* vector species primarily belong to the *Anopheles maculipennis* subgroup (Sinka et al., 2010). Among its 11 Palaearctic sibling species (Linton et al., 2007; Harbach, 2013), *An. atroparvus* (van Thiel, 1927), is the most abundant and widely distributed (Hertig, 2019). This species inhabits coastal and inland areas throughout eastern and central Europe, the Iberian Peninsula and the UK (Sinka et al., 2010; Piperaki and Daikos, 2016). However, its absence has been suggested in Greece, Turkey (Odolini et al., 2012) and partially in southern Italy where it is replaced in coastal areas by *An. lanbranchiae* (Romi et al., 1997). Immature stages of *An. atroparvus* mostly inhabit a variety of permanent or semi-permanent water bodies characterized by clear standing, or slow flowing, brackish and/or fresh water. They are commonly collected along river and lake margins, marshes, irrigation canals and especially in rice fields (primary larval habitat), where aquatic vegetation provides protection from predators and a cooler environment (Jetten and Takken, 1994; Bueno-Mari and Jiménez-Peydró, 2012). *Anopheles atroparvus* has been described as an endophilic, most commonly endophagic, and zoophilic species with a marked preference for domestic farm animals (Bueno-Mari and Jiménez-Peydró, 2010; Lourenço et al., 2011; Martínez de la Puente et al., 2013; Kampen et al., 2016; Brugman et al., 2017). Due to its association to human settlements, *An. atroparvus* also demonstrates anthropophilic behavior (Sinka et al., 2010).

Historically, *An. atroparvus* was implicated in the transmission of local strains of both *Plasmodium vivax* (Bueno-Mari and Jiménez-Peydró, 2012) and *P. falciparum* (Jetten and Takken, 1994). A recent study in which DNA was recovered from historic blood slides of patients infected during the 40's showed that both *P. vivax* and *P. falciparum* were circulating at Ebro Delta (Spain) (Gelabert et al., 2016), an area where *An. atroparvus* is the only anopheline species recorded (Bargues et al., 2006; Gelabert et al., 2016). Moreover, susceptibility tests demonstrated that different European populations were capable of transmitting imported *P. vivax* (Daskova and Rasnicyn, 1982) and *P.*

ovale strains (Garnham et al., 1954), but were, to some degree, refractory to tropical *P. falciparum* strains (Ramsdale and Coluzzi, 1975; Zuleta et al., 1975; Daskova and Rasnicyn, 1982).

Currently, despite the situation that most of the European continent demonstrates "anophelism without malaria" (Jetten and Takken, 1994), significant increases in the number of imported cases (Piperaki and Daikos, 2016), sporadic episodes of local transmission in some countries (Baldari et al., 1998; Kruger et al., 2001; Doudier et al., 2007; Armengaud et al., 2008; Santa-Olalla et al., 2010; Danis et al., 2011; Arends et al., 2013), and predictions that climatic change could increase the risk of malaria transmission (Capinha et al., 2009; Sainz-Elipe et al., 2010; Bueno-Mari and Jiménez-Peydró, 2012; Hertig, 2019) have raised new concerns for the reintroduction of malaria.

To better assess the potential risk of malaria resurgence in Europe, it is necessary to conduct vector competence studies to establish the vector-parasite relationships between local populations of *Anopheles* mosquitoes with the most commonly imported *Plasmodium* species. Consequently, as a first step, the aim of the present study was to establish a laboratory colony of *An. atroparvus* from the Ebro Delta, a former malaria endemic area of Spain, and provide a detailed rearing protocol for further malaria research.

MATERIALS AND METHODS

Study Area

The Ebro Delta is one of the most relevant ecosystems in the Western Mediterranean. It is located in Tarragona Province (Catalonia-Spain) and covers 320 square kilometers. The Ebro River divides the delta plain into two regions, the Baix Ebre from the north, with its capital Tortosa; and the Montsià from the south, with its capital Amposta. The delta is characterized by highly diverse aquatic habitats, e.g., marshes, wetlands, ponds and lakes that co-occur

with densely populated areas and croplands, mostly intended for rice cultivation. The dominance of water systems in the Ebro Delta have favored the proliferation of vector mosquito species, e.g., *An. atroparvus* which was previously incriminated as a primary malaria vector (Sainz-Elipe et al., 2010).

Field Mosquito Collections

To start the laboratory colony, adult anopheline mosquitoes were collected weekly between August and September 2017. In rice growing areas from the municipality of Amposta (40°42′32.5686″N, 0°35′12.2814″E), resting male and female mosquitoes were collected in an unused shed using mouth aspirators (John W. Hock Company, Gainesville, FL, USA), placed in 30 × 30 × 30 cm BugDorm (Bioquip, Rancho Dominguez, CA, USA) insect rearing cages and transported live to the laboratory.

Laboratory Mosquito Rearing Protocol

At the *Institut de Recerca i Tecnologies Agroalimentaries - Centre de Recerca en Sanitat Animal* (IRTA-CReSA) biosafety level 2 facilities (BSL2), a sterile 10% sucrose solution was provided to wild-caught adults by placing a 50 ml glass bottle of the solution containing a filter paper fan for mosquitoes to feed *ad libitum*. Ten percent (10/100) of the captured females were dissected to determine gravid rates. Since all the dissected females were gravid, a Petri dish filled with dechlorinated tap water was placed inside the cages for oviposition. Since no eggs were laid during the first week, several artificial blood meals were offered. Field-collected females were provided blood meals on fresh whole rabbit blood (supplied by a local slaughterhouse) for 3 h at dusk using the Hemotek feeding system (Discovery Workshop, Accrington, UK) set at 37.5 ± 0.5 °C and Parafilm as a feeding membrane. On day 1 post-feeding, a Petri dish containing dechlorinated tap water for oviposition was placed inside the cages and kept until eggs were laid. Egg batches were transferred to sterile plastic trays (22 × 15 × 6 cm) containing 500 ml of dechlorinated and oxygenated tap water. One-fourth Gayelord Hauser Superlevure brewer's yeast

tablet was added to stimulate hatching. To confirm the identity of this mosquito population, 25 wild-caught females (that fed and oviposited) were molecularly analyzed by polymerase chain reaction (PCR) (Proft et al., 1999).

Upon hatching, up to 100 first-instar larvae (L1) were transferred to sterile plastic trays (22 × 15 × 6 cm) containing 500 ml of dechlorinated and oxygenated tap water. Larvae (L1 to L4), were fed 0.1 g minced Tetra Goldfish Flakes and Tetra ReptoMin Sticks (1:1) mixture. Water from rearing trays and food supply were replaced daily.

Pupae were collected daily using a 3 ml plastic pipette and deposited in sterile plastic cups (9 cm in diameter per 7 cm height) containing dechlorinated and oxygenated tap water. Cups containing F1 pupae were placed inside 30 × 30 × 30 cm BugDorm (Bioquip) insect cages with a density of 500 specimens per cage. Adults were provided a 10% sucrose solution *ad libitum* as previously described.

Rearing procedures were followed for subsequent generations with slight modifications: (i) ten day-old (or older) females were deprived sucrose for 48 h and provided blood meals as described above, blood-fed females were placed in a separate cage after feeding; (ii) the oviposition Petri dish with dechlorinated tap water was placed in the cage containing blood-fed mosquitoes at day 5 post-feeding; and (iii) water from larval trays was replaced every 2 days during development. The day the water was not changed, 100 ml of oxygenated and dechlorinated tap water was added to oxygenate and maintain water level. Larval food was added daily.

The lifecycle of *An. atroparvus* mosquitoes was monitored under controlled laboratory conditions simulating field summer conditions of their original habitat (temperature: 25–20 °C for day and night respectively, relative humidity: 80%, and a photoperiod: 12 h light: 11 h dark with two 30 min crepuscular periods).

Colony Assessment

Hatching, larval mortality and feeding rates were calculated and, larval and pupal development times were determined to evaluate laboratory adaptation of the colony. The hatching rate (HR) was calculated as the proportion of L1 larvae/number of eggs. Larval mortality rate (LMR) was calculated as the total number of pupae/L1 larvae. Feeding rate (FR) was calculated as the number of engorged females/the total number of females at the time of blood-feeding. Larval and pupal development times were calculated, respectively, as the number of days between L1 to pupae, and from pupae to adult emergence. Since most comprehensive data were obtained from the second generation (F2), hatching, feeding and mortality rates were calculated from this time point onwards. For larval and pupal development times, data from the fourth generation (F4) onwards were used. The purity of the colony was molecularly verified by PCR (Proft et al., 1999) analyzing 10 females from both, F6 and F10.

RESULTS AND DISCUSSION

An indigenous *An. atroparvus* population from Amposta (the Ebro Delta) was successfully colonized in our laboratory and its rearing protocol standardized. The colony constitutes one of the reference mosquito strains available within the INFRAVEC2 project for vector research.

Approximately 20% of 10-day-old females from generations F2-F6 fed on rabbit blood provided by an artificial (Parafilm) membrane. However, the feeding rates increased up to 45% in later generations (F9) (**Figure 1A**). Eggs were oviposited on day 5 post-blood feeding and eggs hatched after 1–2 days. In early generations (F2-F4), between 48–55% of the eggs hatched, while in later generations, hatching rates increased to 75–92% (**Figure 1B**). The increase in hatching rates reflects the successful adaptation of male-mating

activity as reported for other free-mating culicids (Hoshino et al., 2010; Lima et al., 2004).

Throughout laboratory colonization, a clear reduction in larval mortality was observed, from 70% in F2, to less than 20% in the latest generations (**Figure 1C**), with more than 80% of the first instars reared to adults. The mortality of pupae was almost null in all generations. Both, larval and pupal development times were variable. On average, 13–16 days were required from L1 to pupae (larval development time), and between 1–3 days from pupae to adult emergence (pupal development time) (**Table 1**). A 1:1.14 female:male ratio was observed. Adult lifespan in our laboratory under field-simulated conditions surpassed nine weeks, enough time to conduct vector competence and susceptibility assays. The stenogamic behavior described for Spanish populations (Bueno-Mari and Jiménez-Peydró, 2010) was confirmed in the *An. atroparvus* colony and under laboratory conditions males successfully mated with females in small cages. Swarming and mating events were observed during blood feeding, contradicting previous behavioral descriptions (Jetten and Takken, 1994). Egg development and development times of immature stages observed under the present laboratory conditions were in agreement with previous studies that used similar temperatures (Jetten and Takken, 1994), showing the relevance of this variable during colonization attempts of vector mosquito species.

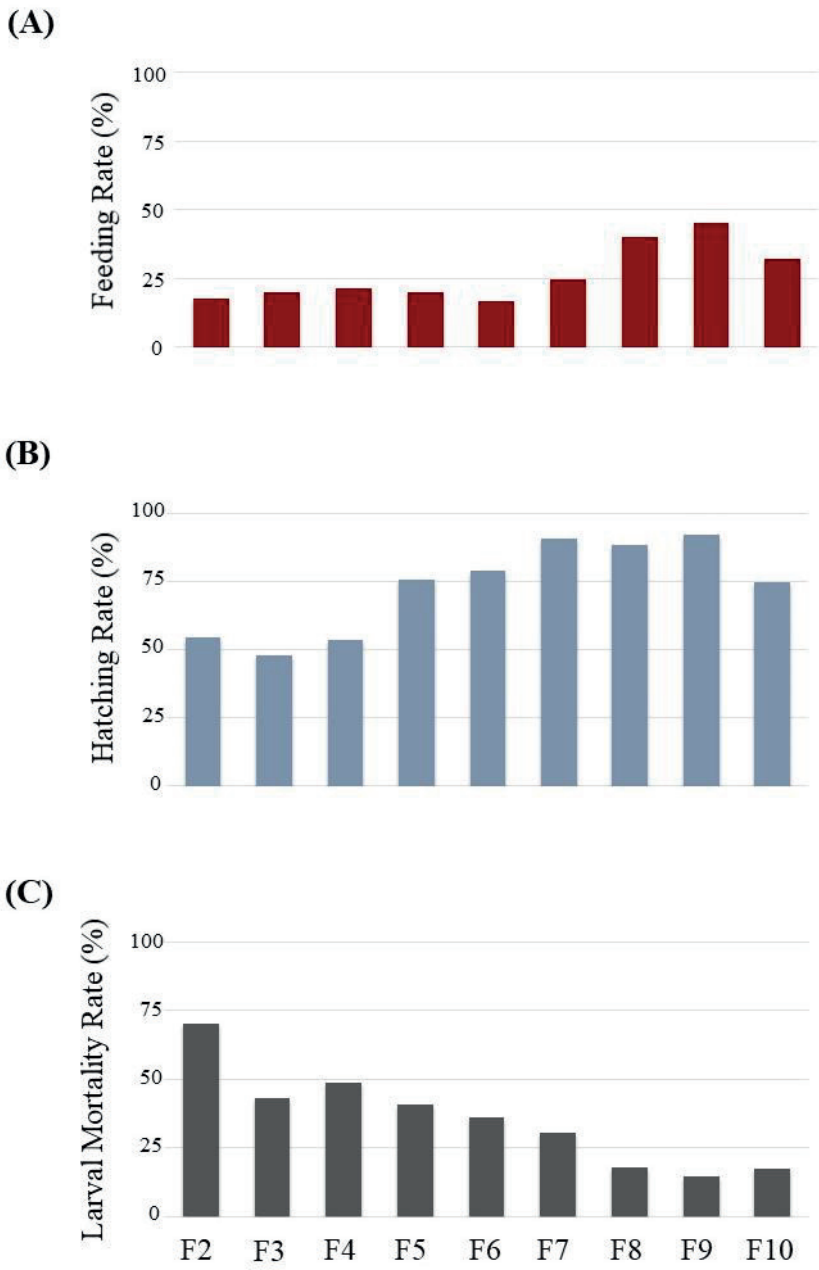

Figure 1. Development of *Anopheles atroparvus* collected from the Ebro Delta under controlled laboratory conditions. **(A)** Feeding rate (FR), engorged females/total number of females at the time of feeding. **(B)** Hatching rate (HR), total number of L1/total number of eggs. **(C)** Larval mortality rate (LMR), total number of pupae/total number of L1.

Table 1. Development times in immature stages of *Anopheles atroparvus* during laboratory colonization

	Development time (days)	
Generation	**Larva to pupa**	**Pupa to adult**
F2	9–25	na
F3	9–25	na
F4	8–22	1–3
F5	12–25	1–4
F6	11–24	1–4
F7	10–26	2–4
F8	8–22	1–3
F9	10–21	1–3
F10	10–18	1–3

Abbreviation: na, data not available

Finally, that the diagnostic PCR methods described by Proft et al. 1999 for the identification of six sibling species of the Maculipennis subgroup resulted in the amplification of three fragments per individual, which corresponded in size to *An. atroparvus* (117 bp), *An. melanoon* (224 bp) and *An. labranchiae* (374 bp). However, after sequencing, all three PCR products corresponded to gene sequences of *An. atroparvus*. Based on our experience (Bargues et al., 2006), *An. atroparvus* is the only anopheline species distributed in this area and these findings suggest that the single 3'-end nucleotide substitution in the primer annealing sites, in the case of *An. melanoon* and *An. labranchiae*, does not provide a unique diagnostic gene fragment for the *An. atroparvus* population studied.

CONCLUSIONS

The present study provides a detailed protocol used to successfully establish and maintain a laboratory colony of a European strain of *An. atroparvus*. Field-caught specimens were only fed via artificial membrane feedings, facilitating the logistics during colony maintenance and during vector competence studies. The potential to evaluate pathogen susceptibility using artificial blood-feeding techniques of earlier laboratory generations would provide a more accurate assessment of vector competence of wild populations.

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ASSAY 2

Microbiota Variation across Life Stages of European Field-Caught *Anopheles atroparvus* and during Laboratory Colonization: New Insights for Malaria Research

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ABSTRACT

The potential use of bacteria for developing novel vector control approaches has awakened new interests in the study of the microbiota associated with vector species.

To set a baseline for future malaria research, a high-throughput sequencing of the bacterial 16S ribosomal gene V3-V4 region was used to profile the microbiota associated with late-instar larvae, newly emerged females, and wild-caught females of a sylvan *Anopheles atroparvus* population from a former malaria transmission area of Spain. Field-acquired microbiota was then assessed in non-blood-fed laboratory-reared females from the second, sixth, and 10th generations.

Diversity analyses revealed that bacterial communities varied and clustered differently according to origin with sylvan larvae and newly emerged females distributing closer to laboratory-reared females than to their field counterparts. Inter-sample variation was mostly observed throughout the different developmental stages in the sylvan population. Larvae harbored the most diverse bacterial communities; wild-caught females, the poorest. In the transition from the sylvan environment to the first time point of laboratory breeding, a significant increase in diversity was observed, although this did decline under laboratory conditions. Despite diversity differences between wild-caught and laboratory-reared females, a substantial fraction of the bacterial communities was transferred through transstadial transmission and these persisted over 10 laboratory generations. Differentially abundant bacteria were mostly identified between breeding water and late-instar larvae, and in the transition from wild-caught to laboratory-reared females from the second generation. Our findings confirmed the key role of the breeding environment in shaping the microbiota of *An. atroparvus*. Gram-negative bacteria governed the microbiota of *An. atroparvus* with the prevalence of proteobacteria. *Pantoea*, *Thorsellia*, *Serratia*, *Asaia*, and *Pseudomonas* dominating the microbiota associated with wild-caught females, with the latter two governing the communities of laboratory-reared females. A core microbiota was identified with *Pseudomonas* and *Serratia* being the most abundant core genera shared by all sylvan and laboratory specimens.

Overall, understanding the microbiota composition of *An. atroparvus* and how this varies throughout the mosquito lifecycle and laboratory colonization paves the way when selecting potential bacterial candidates for use in microbiota-based intervention strategies against mosquito vectors, thereby improving our knowledge of laboratory-reared *An. atroparvus* mosquitoes for research purposes.

Keywords: *Anopheles atroparvus*, field-caught, laboratory colonization, 16S rRNA, microbiota, European mosquitoes.

BACKGROUND

Microorganisms that permanently or transiently reside in mosquitoes are collectively known as microbiota (Villegas and Paolucci, 2014). Bacteria (commensal and/or endosymbiotic), protists, viruses, and fungi, which are the main representatives of this consortium, can be horizontally acquired (i.e., venereal transmission, sharing of environmental/food sources) and/or maternally transferred (Bian et al., 2013; Eleftherianos et al., 2013; Gendrin and Christophides, 2013; Bili et al., 2016). Despite being found colonizing the mosquitoes' midgut epithelia, hemolymph, salivary glands, and gonads (Dillon and Dillon, 2004; Minard et al., 2013; Villegas and Paolucci, 2014), the midgut microbiota has been the most extensively studied. The midgut microbiota is primarily shaped by the environment (Gendrin and Christophides, 2013; Dennison, Jupatanakul and Dimopoulos, 2014; Hegde et al., 2018) and varies dynamically throughout the mosquito's life cycle (Duguma et al., 2015). During larval development, immature stages ingest organic matter, detritus, and microorganisms from their aquatic habitat (Merrit, Dadd and Walker, 1992) and acquire a considerable fraction of their microbiota (Wang et al., 2011). Only those microbes that withstand and adapt to the midgut's microhabitat could be passed through transstadial transmission, from larvae to adults, and may persist in mosquito populations as part of the indigenous microbiota (Pumpuni et al., 1996). In adult mosquitoes, diverse dietary regimes (e.g., plant sap and nectar or blood) may alter the composition of the microbiota and incorporate diversity into the microbial communities (Rani et al., 2009). In fact, descriptive studies that have characterized the microbiota of several field populations and/or laboratory colonies of culicid mosquitoes have suggested geographical, species, sex, and even individual variation (Yadav et al., 2015; Akorli et al., 2016; Muturi et al., 2016; Bascuñan et al., 2018; Rodriguez-Ruano et al., 2020; Saab et al., 2020; Tainchum et al., 2020; Silva et al., 2021).

The microbiota plays an essential role in relevant physiological traits of diverse vector mosquitoes, such as larval development (Chouaia et al., 2012; Coon et al., 2014; Martinson and Strand, 2021), mosquito lifespan (McMeniman et al., 2009; Wei et al., 2017; Mancini et al., 2020), fecundity, and blood digestion (Gaio et al., 2011; Gnambani et al., 2020). Moreover, microbiota has been involved in both infection susceptibility of the mosquito and vector competence. However, its role and mechanisms are diverse and extensive with some commensal bacteria and/or endosymbionts able to produce "anti-pathogen molecules" or activate cross-reactive innate immune responses (Dong, Manfredini and Dimopoulos, 2009; Moreira et al., 2009; Cirimotich et al., 2011; Ramirez et al., 2012; Bai et al., 2019).

In recent years, vector-borne disease research has focused its efforts on studying multiple aspects of mosquito–microbiota–pathogen interactions for the development of novel and more effective control strategies. Conventionally, in such studies, laboratory breeding of mosquito vectors has been a useful tool for obtaining large numbers of experimental individuals and controlling experimental conditions (Romoli and Gendrin, 2018). However, outcomes may not necessarily represent what might occur in the wild (Akorli et al., 2019) due to changes in the fitness of the mosquito and its immune system as consequence of the reduction in microbial diversity as previously reported in laboratory-colonized specimens (Rani et al., 2009; Duguma et al., 2015; Dada et al., 2020). Therefore, a better understanding of field-acquired microbiota during the laboratory colonization of sylvan mosquito populations is essential to set baselines for functional studies.

On the European continent, sibling species of the *Anopheles maculipennis* subgroup are considered the primary vectors of *Plasmodium* parasites, which are the causative agents of malaria (Sinka et al., 2010). Among them, *Anopheles atroparvus* van Thiel, 1927 is still one of the most widely distributed species, capable of transmitting local strains of both *Plasmodium vivax* and *Plasmodium falciparum* (Jetten and Takken, 1994; Bueno-Mari and Jiménez-Peydró, 2012), as

well as imported strains of *P. vivax* (Daskova and Rasnicyn, 1982) and *Plasmodium ovale* (Garnham et al., 1954). Following the eradication of malaria from Europe, the study of its vectors suffered substantial decrease, accompanied in turn by a subsequent information gap on the biology of local populations of *Anopheles* mosquitoes. Currently, sporadic outbreaks of autochthonous malaria transmission (Santa-Olalla et al., 2010; Danis et al., 2011; Arends et al., 2013), in addition to an increase in the number of imported cases (Piperaki and Daikos, 2016) and the prognostics of resurgence in the continent due to globalization and climate change (Hertig, 2019), have awakened new interests in their study.

To date, the microbiota of *An. atroparvus* has not been analyzed, and for this reason, taking into consideration malarial research in Europe, the present work aimed to (i) identify the bacterial communities associated with a sylvan Mediterranean population of *An. atroparvus* and (ii) assess the influence of laboratory breeding on the structure (diversity and composition) of the mosquito's natural microbiota. To accomplish these goals, the microbiota profile of a local population of *An. atroparvus* from the Ebro Delta, a former malaria transmission area of Spain, was characterized using high-throughput sequencing of the bacterial 16S ribosomal gene V3-V4 region. Bacterial community composition was identified in late-instar larvae, newly emerged females, and field-caught females. The contribution of water from the natural breeding site to the microbial diversity of this anopheles population was evaluated. Finally, the composition of the microbial communities was assessed throughout the laboratory colonization process, from both the sylvan population and those over the second, sixth, and 10th generations produced under controlled laboratory conditions.

MATERIALS AND METHODS

Experimental Design

To characterize the microbiota of an *An. atroparvus* population in its original habitat and to then evaluate its evolution over 10 generations under controlled laboratory conditions, four time points were set: sylvan (T0), and second (F2), sixth (F6), and 10th (F10) generation produced in the laboratory. From the sylvan environment, third- and fourth-instar larvae (L), newly emerged females (E), and wild-caught females (F0) were sampled. From laboratory, F2, F6, and F10 7- to 9-day-old females that had been sugar-fed (sterile 10% sucrose solution *ad libitum*), but had never been blood-fed, were selected. To identify the contribution of breeding water to the bacterial community composition in this mosquito population, water from the natural breeding site (W) was sampled.

Sample Collection and Processing for Microbiota Characterization

From July to September 2017, rice paddies in the municipality of Amposta (Ebro Delta – Spain) (40° 42' 32.5686"N, 0° 35' 12.2814"E) were visited once a week for the collection of immature stages and adult indoor catches. Late-instar larvae (L3-L4) and pupae were collected using the conventional dipping technique and were transported live to the laboratory in sterile plastic containers with water and substrate from their original breeding site. Additional water samples were collected at the same depth where larvae were found and transported to the laboratory in sterile plastic containers at 4 ºC for preservation. Female and male anophelines were captured inside an unused shed using mouth aspirators (John W. Hock Company, Gainesville, FL, USA) and placed inside sterile 30 × 30 × 30 cm BugDorm (BioQuip, Rancho Dominguez, CA, USA) insect rearing cages for transportation. At the entomology laboratory from the *Institut de Recerca i Tecnologia Agroalimentaries – Centre de Recerca en Sanitat Animal* (IRTA-CReSA) in Barcelona, wild-caught mosquitoes were

colonized in the laboratory and bred as previously described (Birnberg et al, 2020).

To generate the samples for microbiota analysis (**Table 1**), i) larvae were pooled, ii) pupae were transferred to mosquito breeders (BioQuip, Rancho Dominguez, CA, USA) containing water and substrate from their breeding site for adult emergence – only newly emerged females from the first 48 hours were used, and iii) wild-caught, second, sixth and 10th generation females were frozen and pooled according to origin. Water samples were preserved at -80 ºC until DNA isolation.

Table 1. Sample selection per time point for microbiota analysis of *Anopheles atroparvus* from the Ebro Delta and along laboratory colonization.

Time Point	Sample Type	Abbreviation	No. Pools	No. Specimens per pool or Volume (ml)
T0	Water from breeding sites	W	3	100
	Larvae (L3 – L4)	L	3	20
	Newly emerged females	E	3	20
	Adult field females	F0	3	20
F2	Adult females (2nd lab generation)	F2	3	20
F6	Adult females (6th lab generation)	F6	3	20
F10	Adult females (10th lab generation)	F10	3	20

Notes: F2, F6, and F10 correspond to 7- to 9-day-old females that had been sugar-fed (sterile 10% sucrose solution *ad libitum*), but had never been blood-fed.

To eliminate any possibility of contamination during specimen handling, samples were surface sterilized as follows: first, one rinse in sterile water for one min, two consecutive washes in 70% ethanol for 5 min, one 5-min wash in

a 10-fold dilution of commercial bleach [active chlorine (37 g/l initial concentration]), and a final rinse in sterile water for 1 min. Adult females were sterilized individually, while larvae were sterilized in pools. All samples were preserved at -80ºC until molecular processing.

DNA Extraction from Water Samples and, Larvae and Mosquito Pools

Firstly, larvae and mosquito pools were homogenized using zirconia beads in a FastPrep-24™ 5G (MP Biomedicals GmbH, Eschwege, Germany) bead beating grinder and lysis system. Then, genomic DNA from these samples was isolated with the QIAampDNA Mini Kit (Qiagen, Hilden, Germany) following the protocol for Gram-positive bacteria in which lysozyme (Sigma) was added for enzymatic lysis. DNA from breeding water was extracted using the DNeasy PowerWater Sterivex Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was purified using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and its quality and concentration evaluated using the NanoDrop spectrophotometer (ThermoScientific, Waltham, MA, United States).

16S Ribosomal RNA Gene Sequencing and Bioinformatics Analysis

To generate sequencing libraries, the hypervariable region V3-V4 of the bacterial 16S ribosomal RNA (rRNA) gene was amplified (Klindworth et al., 2013) and the Illumina 16S metagenomics sequencing library preparation protocol (Illumina, Inc., San Diego, CA, United States) was followed. The quality of all libraries was verified individually using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, United States), normalized, and equimolarly pooled in a single library pool. On an Illumina MiSeq platform, samples were paired-end sequenced using the MiSeq Reagent Kit v3 (2 × 300 cycles). Nuclease-free water and theand the ZymoBIOMICS™ Microbial Community Standard (ZYMO Research corp., Irvine, CA, USA) were included as contamination and amplification controls, respectively, and treated as regular samples. Raw sequencing datasets retrieved by this study were deposited in the

National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the Bioproject #PRJNA660574.

After sequencing, raw reads (R1/R2) were merged in PEAR V.0.9.1 software (http://www.exelixis-lab.org/web/software/pear) applying the default parameters and specifying a 70-nt sequence overlap on each end. Then, adapters were identified and removed from the merged sequences using Cutadapt v1.8.1 (Martin, 2011) and sequences shorter than 100 bp were eliminated from the dataset to reduce erroneous taxonomic associations. Finally, low-quality sequences (*phred* score lower than Q20) were eliminated using the BBMap v38 Reformat package. After quality filtering, chimera sequences were identified and eliminated in "Uchime" (Edgar et al., 2011). To assemble operational taxonomic units (OTUs), good-quality sequences with at least 97% similarity were clustered in the Cluster Database at High Identity with Tolerance (cd-hit) software v2.6.8 (Li et al., 2002). For OTU annotation, assemblies were compared to the 16S rRNA gene sequence reference (RefSeq) database of the NCBI and the closest hit was reported. Taxonomy summaries with relative abundances at phylum, family, genera, and species levels were generated.

Data Analyses

Diversity analyses, ordination methods, and differential analyses for microbiota composition were performed in "R" v3.6.0 (R Core Team, 2016) statistical software. Prior data analyses, spurious OTUs (one sequence present in only one sample) were eliminated, and count matrices were rarefied at 20,465 sequences per sample using the "phyloseq" package as previously described (Weiss et al., 2017). In the "vegan" package for community ecological analyses (Okasen et al., 2020), alpha diversity was estimated by calculating the microbial/OTU richness and Shannon and Simpson indices. To assess the variation between sample types (i.e., breeding water (W), larvae (L), newly emerged (E) and wild-caught (F0) females, and second (F2), sixth (F6), and 10th (F10) generation laboratory females), an analysis of variance (ANOVA) was conducted. Differences

between bacterial communities among sample types were evaluated by a principal coordinate analysis (PCoA) based on a Bray–Curtis dissimilarity matrix, and the significance of these associations was tested with a PERmutational Multivariate ANalysis Of VAariance (PERMANOVA) using 1,000 permutations. Data distribution was visualized in "ggplot" (Wickham, 2009). To measure and compare the uniqueness of bacterial communities from each sample type and assess their input to the diversity between groups, a local contribution to beta diversity (LCBD) test was executed (Legendre and De Cáceres, 2013). For differential abundance analysis, in the "DESeq2" package (Love et al., 2014), a generalized linear model (GLM) for fixed effects was generated using the negative binomial family between pairs of samples (W/L, L/E, E/F0, F0/F2, F2/F6, and F6/F10). Then, a Wald test was performed and the Benjamini and Hochberg false-discovery rate (FDR) correction was used for *p-value* adjustment (Benjamini and Hochberg, 1995). Bacterial taxa present in at least 50 % of the samples of each group (i.e., W, L, E, or Fx) with an average number of normalized sequences (BaseMean) higher than 10 that presented an adjusted *p-value* lower than 0.05 were considered as differential taxa. The logarithm 2 of the relative change (log_2FC) of each bacterial group at the genus level was calculated to estimate the abundance of differential bacteria per pair of samples. To determine the contribution of natural breeding water to the microbiota of *An. atroparvus* and identify the bacteria that may persist as a result of transstadial transmission and/or over 10 generations under controlled laboratory conditions, the unrarefied OTUs were used to identify shared and unique genera. Common genera between two sample types, which were present in at least one pool (out of three) from each group, were considered as "shared." Sample interactions were then represented with Venn diagrams. Finally, to describe the core microbiota, meaning the bacteria stably associated with a certain mosquito species in different mosquito stages (i.e., L, E, F0–F10), those genera identified in two pools out of three with at least 10 reads per each sample type were selected.

RESULTS

Sequencing Data Output Summary and Taxonomic Assignations

High-throughput sequencing of the bacterial 16S rRNA gene V3-V4 region of *An. atroparvus* (sylvan and laboratory) and its natural breeding water generated a total of 1,364,231 raw reads. After quality filtering and chimeric sequence removal, reads per sample ranged from 20,465 to 107,148. In total, 1,082,199 clean sequences were used to assemble 20,462 different OTUs, of which 80% were successfully annotated and distributed into 24 phyla, more than 300 families, and nearly 1200 genera (**Supplementary File 1**). At phylum level, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, and *Cyanobacteria* accounted for 94% of the total microbiota. *Proteobacteria* was identified as the most abundant phylum gathering, by itself, 52% of the overall OTUs (**Supplementary Figure 1A**). At lower taxonomic levels, OTUs were distributed in several low abundant taxa with *Pseudomonadaceae* (7%), *Flavobacteriaceae* (4%), *Comamonadaceae* (4%), and *Acetobacteraceae* (4%) being the most abundant families and *Pseudomonas* (6%), *Asaia* (4%), and *Flavobacterium* (3%) being the most representative genera (**Supplementary Figures 1B, C**). Rarefaction curves in almost all samples, except for water, reached the plateau, implying that most of the bacterial diversity was captured (**Supplementary Figure 2**).

Negative and positive controls yielded 15 and 10,000 sequences, respectively. Since the few OTUs from the negative control exhibited low identities and none of these were detected in any of the studied samples, laboratory contamination was discarded. Likewise, since only the expected bacteria were identified in the microbial standard, taxonomic outcomes were verified.

An. atroparvus Immature Stages Harbor More Diverse Bacterial Communities than Adult Females

Diversity indices revealed that the structure (diversity and composition) of bacterial communities in breeding water, as well as those in sylvan and laboratory-reared *An. atroparvus*, varied according to group of origin (i.e., breeding water (W) larvae (L), newly emerged (E) and wild-caught (F0) females, and second (F2), sixth (F6), and 10th (F10) generation laboratory females). Pairwise ANOVA comparisons of OTU richness and Simpson (1-D) and Shannon (H) indices provided first-hand evidence of this variation. At all taxonomic levels analyzed (i.e., family, genera, and species) (**Figure 1** and **Supplementary Figures 3, 4**), OTU richness was significantly higher in breeding water (W) than in larvae (L) and adult mosquito samples (E and F0–F10) ($p < 0.05$). Within sylvan and laboratory environments, significant differences were found between L and F0 ($p = 0.001–0.01$) and between F2 and F10 ($p = 0.001–0.05$), respectively. Simpson and Shannon indices showed that, among the studied biological samples, L was the most diverse and evenly distributed, while F0 and F10 were the least diverse and highly uneven samples. It is noteworthy that statistical differences were only identified within the sylvan environment between L and E (Simpson: $p = 0.001–0.05$; Shannon; $p = 0.01–0.05$) and between L and F0 (Shannon: $p = 0.01–0.05$), while no differences were found between laboratory time points (F2–F10). A significant variation was observed in the transition F0/F2, from wild-caught females to the first time point under laboratory conditions (OTU richness: $p < 0.05$; Shannon: $p = 0.01–0.05$).

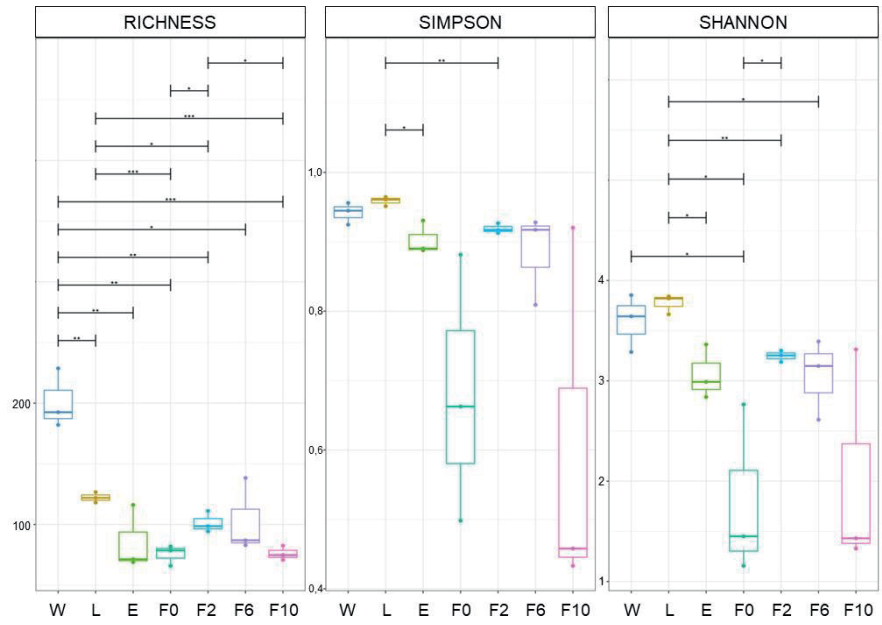

Figure 1. Differences in bacterial community structure. OTU richness and Simpson and Shannon indices estimated at family level. Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively. Boxes represent the interquartile range within each group. The line that divides the box corresponds to the median and dots, to minimum and maximum scores. Analysis of variance (ANOVA) significance levels: **p* = 0.01–0.05; ***p* = 0.001–0.01; ****p* < 0.001.

Principal coordinate analyses (PCoA) also evidenced diversity variation among different types of samples and for all taxonomic levels. Spatial distribution and the low variance explained by the first two dimensions (43.5–51.5%) indicated that bacterial communities clustered differently according to origin (i.e., W, L, E, F0–F10). The significance of this differential segregation was further confirmed by PERMANOVA (1,000 permutations; $p < 0.001$; R2 = 0.53–0.57) (**Figure 2A** and **Supplementary Figures 5A, 6A**). When observing ordination plots, it is worth noting that all biological samples (sylvan and laboratory) distributed distantly from natural breeding water (W), suggesting a more unique microbiota composition in the latter, a fact corroborated by LCBD analysis (**Figure 2B** and **Supplementary Figures 5B,**

6B). In addition, larvae (L) and newly emerged females (E) distributed closer to laboratory-reared females (F2–F10) than to wild-caught females (F0), implying that, despite their sylvan origin, their microbiota was more similar to that of laboratory females than that of their sylvan counterparts (F0). Furthermore, the heterogeneity previously observed in F0 and F10 (**Figure 1**) was supported by the extended confidence ellipses shown in the PCoA and by LCBD analysis, which identified these bacterial communities (together with breeding water) as major contributors to the observed diversity differences between sample types (**Figure 2B** and **Supplementary Figures 5B, 6B**).

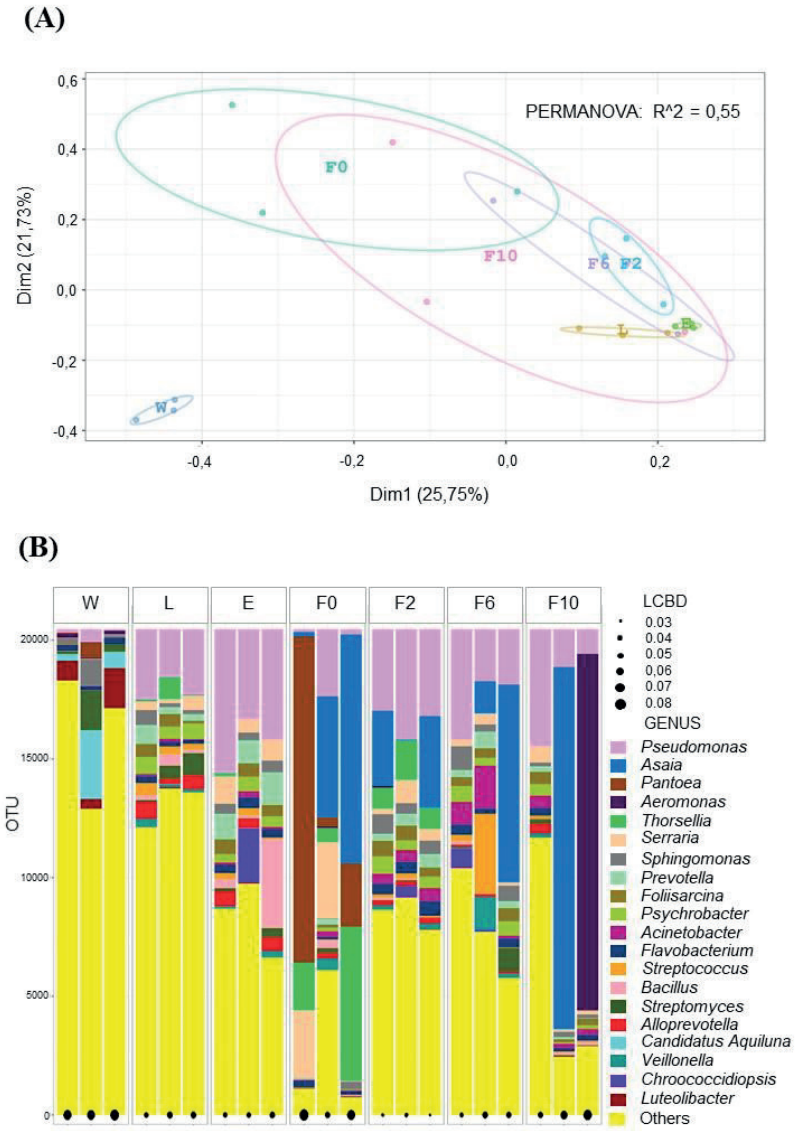

Figure 2. Beta diversity analyses at genus level depicted microbial community variation. PCoA plot showing bacterial community clustering and segregation according to origin. Color points represent the microbiota of a pool of 20 individuals and color ellipses represent confidence intervals per sample type (**A**). Local contribution to beta diversity analysis (LCBD) showing the uniqueness of bacterial community composition per pool per sample type. The measure of the input is given the size of the black dot (e.g., the larger the dot, the more unique the microbial community) (**B**). Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively.

The Microbiota of *An. atroparvus* is Governed by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*

Taxonomic identification of sequences depicted that the microbiota profile of different sample types analyzed was primarily shaped by the same taxa, although with different proportions (**Figure 3**). For instance, at phylum level, *Proteobacteria* dominated all bacterial communities with relatively high abundance ranging from 44% in larvae (L) to 89% in wild-caught females (F0). While *Actinobacteria* was the second most abundant phylum in W (22%) and L (18%), it dropped to the third/fourth position in adult females (E and F0–F10) accounting for less than 8% of microbiota. Similarly, whereas *Verrucomicrobia* and *Planctomycetes* belonged to the top five phyla in W, with abundances of 9 and 5%, respectively, these were barely detected in biological samples (<1%). In addition, phyla detected in our studied samples, such as *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria*, also fluctuated between different types of samples below 18, 14, and 8%, respectively (**Figure 3A**). At lower taxonomic levels, the same trend was observed, and few dominant taxa were identified across sample types. *Pseudomonas* (*Pseudomonadaceae* family), for example, was present in all microbial communities with a high prevalence in biological samples and high abundance ranging from 13% in L and laboratory-reared females at the 10th generation (F10) to 25% in newly emerged females (E). Together with *Pseudomonas*, *Asaia* (*Acetobacteraceae* family) governed the microbiota of laboratory-reared females with frequencies of 12, 17, and 25%, respectively, for F2, F6, and F10; however, in F0, *Pseudomonas* was poorly represented (5%) and in L and E, *Asaia* was scarce (<1%). Besides *Asaia*, the microbiota of F0 was also dominated by *Pantoea* (*Erwiniaceae* family) (27%), *Thorsellia* (*Thorselliaceae* family) (15%), and *Serratia* (*Yersiniaceae* family) (10%), genera that were less frequent in the other sample types (**Figures 3B, C**).

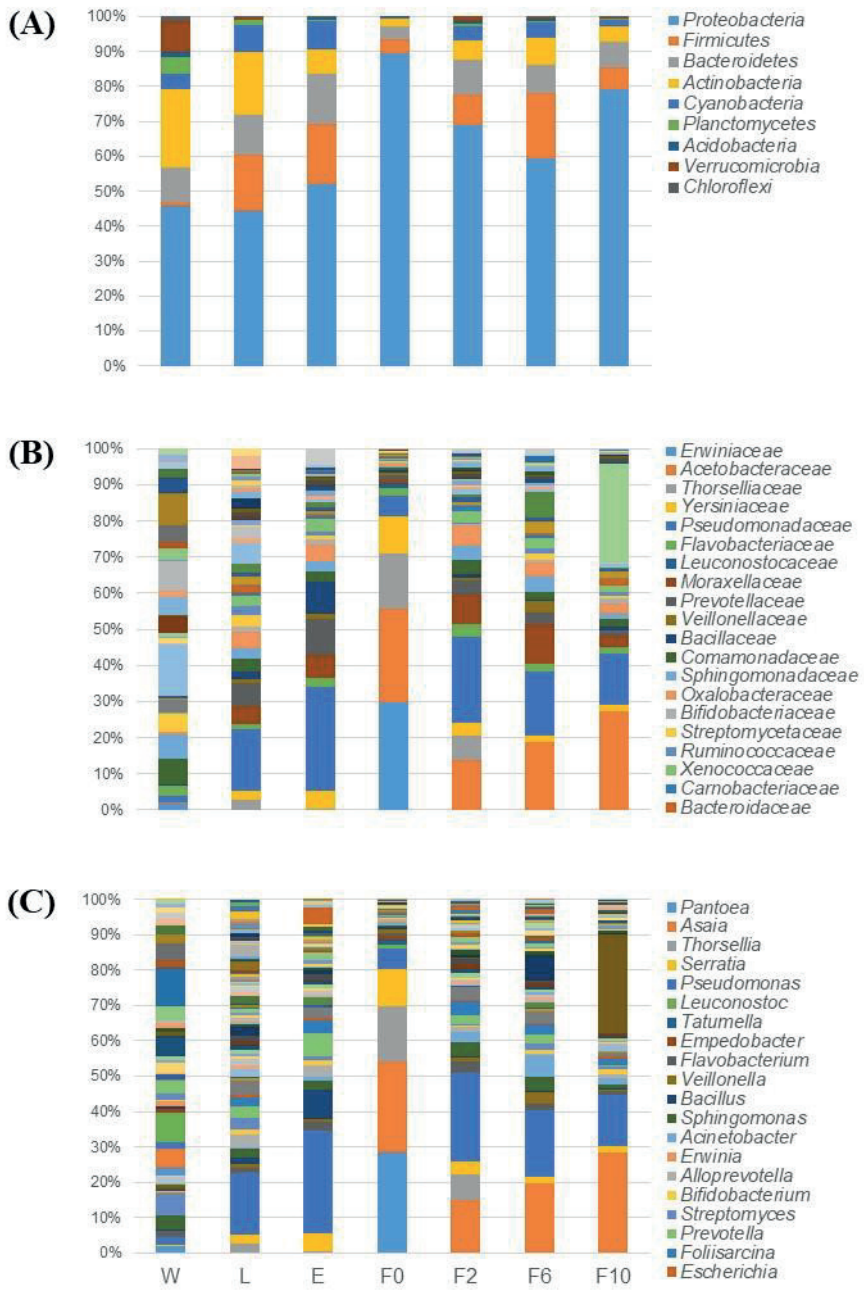

Figure 3. Microbiota composition in breeding water and in sylvan and laboratory-reared An. atroparvus at phylum (**A**), family (**B**), and genus (**C**) levels. The average of the relative abundances per bacterium from three pools per sample type is represented in bars, and the top 20 taxa are shown.

For a more comprehensive profiling, the dominant genera were analyzed at a finer taxonomic level. All the reads associated with *Asaia* belonged to a single species, *Asaia siamensis*, with its most abundant OTU (OTU_19275) being detected in all sample pools including breeding water. Likewise, *Thorsellia anophelis* was the only species recognized with OTU_1682 being found in at least 2 out of three pools of all sylvan samples (including water) and F2. Reads associated with several species of *Pantoea*, *Pseudomonas*, and *Serratia* were identified, although only one OTU assigned to *Pantoea deleyi* (OTU_347) was present in 2 out of three pools of sylvan samples and one OTU assigned to *Pseudomonas migulae* (OTU_107) detected in all sample pools. Lastly, *Serratia liquefaciens* (OTU_1700) was frequently high in almost all sample pools whereas *S. marcescens* was found only in sylvan samples and majorly in F0.

The Microbiota of *An. atroparvus* is Acquired Mostly from its Natural Breeding Water and Can Persist throughout Different Sylvan Life Stages and Over Laboratory Colonization

Pairwise, microbiota comparisons unveiled a considerable fraction of common bacteria between subsequent sample types (**Figure 4**). Within the sylvan environment, when natural breeding water (W) was contrasted with late-instar larvae (L), more than three-quarters (77%; 340/442) of the bacterial genera detected in L were shared with the water where they developed. Likewise, 67% (207/309) of the genera found in newly emerged females (E) were present in L and 48% (134/278) of bacteria inhabiting wild-caught females (F0) were also identified in E. In the transition from sylvan to laboratory environments, 59% (163/278) of the microbiota found in F0 was recovered in females from the second generation produced under controlled laboratory conditions (F2). During the laboratory colonization process, females from the sixth (F6) and 10th (F10) generation shared 65% (256/394) and 72% (192/268) of their microbiota with their previous time point, F2 and F6, respectively (**Figure 4A**). It is noteworthy that most bacteria inhabiting F0 and F10 were also identified

in natural breeding water (**Figure 4B**) emphasizing the contribution of the aquatic habitat to the microbiota composition of adult mosquitoes. When sylvan and laboratory samples were compared as a whole, only a small proportion of bacteria were unique to laboratory (**Figure 4C**). In addition, more than half of the bacteria were unique to the sylvan environment with 64% (398/625) of these bacteria being exclusive to natural breeding water; in larvae, newly emerged and wild-caught females less than 5% of their microbiota was unique.

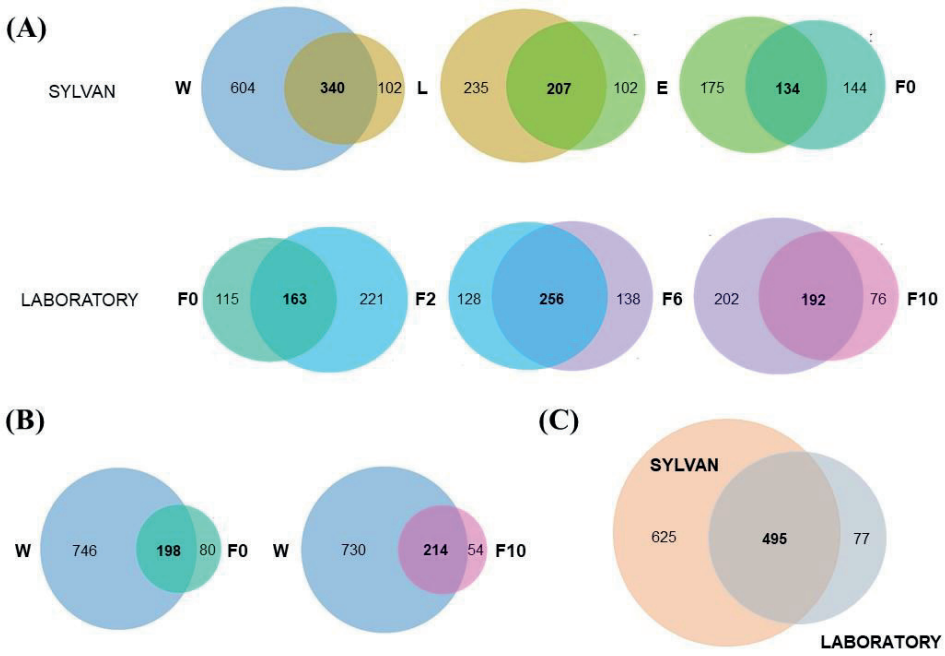

Figure 4. Microbiota of *An. atroparvus* persists across sylvan samples and laboratory time points. Venn diagrams showing the number of shared genera (present in at least one pool, out of three, of both groups) among subsequent pairs (**A**), between natural breeding water with wild-caught females and with laboratory-reared females from the 10th generation (**B**), and between sylvan and laboratory environments (**C**). Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively.

Finally, through differential abundance analysis, a small fraction of bacteria was considered differentially abundant when subsequent pairs were analyzed (i.e., W/L, L/E, E/F0, F0/F2, F2/F6, and F6/F10). The highest

numbers were obtained in transitions W/L (from natural breeding water to late-instar larvae) and F0/F2 (from wild-caught females to laboratory-reared females from the second generation) with 105 and 55 (out of 1197) differential genera, respectively (**Supplementary File 2**).

The Core Microbiota of *An. atroparvus* is Dominated by Few Bacteria

Overall, 22 (out of 1197) bacterial genera were recognized as part of the core microbiota of *An. atroparvus* (**Figure 5**) with *Pseudomonas* and *Serratia* being the most representative genera shared by immature stages (L) and adult females (E, F0–F10). Thirteen of the core genera in *An. atroparvus* were found in both breeding water and biological samples indicating that these bacteria could have been environmentally acquired. In contrast, the remaining nine were already part of the indigenous microbiota and vertically transmitted.

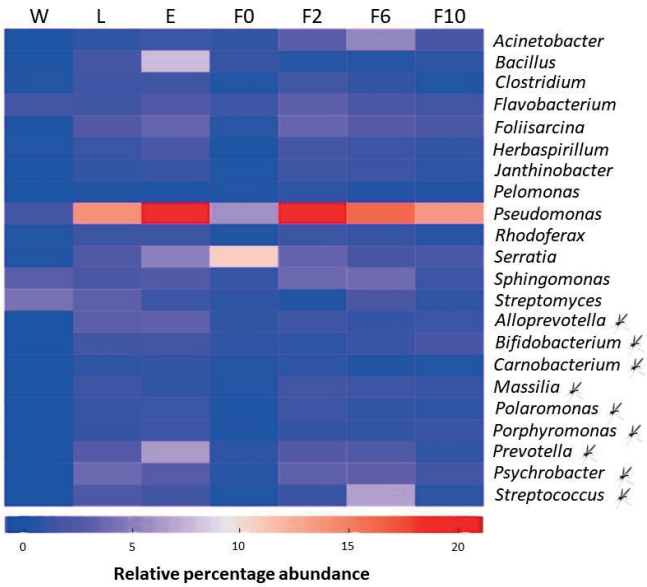

Figure 5. Core microbiota of *An. atroparvus*. Heatmap showing the relative abundance of bacterial genera identified in two out of three pools per sample type with more than 10 reads that are common to all mosquito samples. Genera marked with a mosquito represent core bacteria present in larvae and adult females but not in natural breeding water. Genera without the mark represent core bacteria detected in all sample types. Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively.

DISCUSSION

To set a baseline for future malaria research in *An. atroparvus*, the present study reports, for the first time, the microbiota profile of a sylvan mosquito population from a former malaria transmission area of Europe and assesses field-acquired microbiota along laboratory breeding. Sequencing of the V3-V4 region of the bacterial 16S rRNA gene provided a comprehensive description of bacterial communities and their dynamics across different developmental stages throughout the mosquito's life cycle and during laboratory colonization under controlled conditions. Our data revealed marked inter-sample variations mostly between sylvan life stages, in the transition from sylvan to laboratory environments, and between the first and last laboratory time points. Overall, these findings suggested that the microbiota of *An. atroparvus* was highly influenced by its breeding habitat (i.e., sylvan or laboratory) and metamorphic processes.

Under natural conditions, throughout their life cycle, mosquitoes are in continuous contact with countless sources of microbes, as well as with unstable extrinsic factors (e.g., temperature, droughts, or heavy rains) that may play a role in shaping their microbiota. *Anopheles* mosquitoes, as holometabolous insects, undergo different developmental stages until complete metamorphosis and so exploit different habitats so as to avoid intraspecific competition (Moran, 1994). In our case, larvae of *An. atroparvus* develop just beneath the water surface of permanent or semi-permanent rice paddies, while adults inhabit terrestrial habitats near domestic animals and human dwellings (Birnberg et al., 2020). Consistent with previous reports in different *Anopheles* and *Aedes* species, water from the aquatic habitat from where larvae were collected exhibited the largest OTU richness, while larvae harbored a higher bacterial diversity than newly emerged and adult females (Wang et al., 2011; Dada et al., 2014; Bascuñan et al., 2018; Alfano et al., 2019). Since immature anophelines are filter feeders, bacteria suspended in the aquatic habitat enter into the gut lumen along with the water intake. Thus, as expected, a substantial fraction of the

microbiota recovered in larvae of *An. atroparvus* was present in the water where they develop, confirming high contribution of the aquatic breeding habitat to the microbial community structure in immature stages. Differences in bacterial community structure (diversity and composition) between larvae and their natural breeding water indicated that the larval lumen was the first selective environment for bacteria from the aquatic habitat. While peritrophic matrices work as a physical barrier, the conjunction of the midgut's physio-chemistry and digestive enzymes, host immune response, and competition with indigenous microorganisms generate a challenging microhabitat in which only a subset of bacteria is able to survive (Engel and Moran, 2013). Microorganisms that withstand and colonize the larval midgut are presumed to offer functional advantages to their hosts (Gimonneau et al., 2014). For instance, *Actinobacteria*, which are environmentally derived bacteria, were highly prevalent in *An. atroparvus* larvae and persistent in the adult population. Due to the association of *Actinobacteria* to plant biomass decomposition in aquatic environments (Lewin et al., 2017), these bacteria could be associated with *An. atroparvus* nutritional functions as suggested for other anophelines from Colombia (Bascuñan et al., 2018).

In the transition from aquatic to terrestrial habitats, metamorphosis from larvae to adults involves selective processes that modify the structure of the microbiota. During the ecdysial process, the egestion of the meconial peritrophic matrices (MPMs) and the eventual ingestion of exuvial fluid (with its antiseptic properties) clear the midgut content (Moll et al., 2001), drastically reducing the microbial communities (Wang et al., 2011). Accordingly, the shift from larvae to newly emerged females in *An. atroparvus* resulted in a significant diversity loss, albeit a fraction of the bacterial communities persisted and shared by both developmental stages. This finding is in agreement with previous studies that have analyzed the microbiota dynamics throughout the life cycle of several mosquito populations and reported also in microbial persistence among subsequent stages, suggesting bacterial transstadial

transmission (Rani et al., 2009; Coon et al., 2014, 2016; Gimonneau et al., 2014). In our study, bacterial persistence could have implied one and/or a combination of the following phenomena: (i) an incomplete egestion of MPMs (Moll et al., 2001) and (ii) MPMs that were still present in newly emerged females, due probably to the age of the studied specimens. Data herein reported derived from 0- to 48-h-old newly emerged females and the disappearance of MPMs in *Anopheles* mosquitoes has been seen to occur 16–20 h after emergence (Romoser et al., 2000). (iii) Part of the bacteria could have been reacquired by newly emerged females by imbibing water during hatching (Lindh et al., 2008), since pupae from which *An. atroparvus* females emerged were maintained in their original breeding water. (iv) Bacteria that were transmitted by transstadial means colonized other tissues that are not affected by the potential antibacterial effect of the exuvial (molting) fluid, which may be ingested during metamorphosis (Moll et al., 2001). The high overlap between the bacterial communities in larvae and newly emerged *An. atroparvus* females, which had not been sugar fed, reflected the contribution of the larval aquatic environment to adults' microbiota as previously reported for other anophelines (Akorli et al., 2016), highlighting the relevance of microbial transstadial transmission in shaping the community structure of adult *An. atroparvus* females. Aside from the influence of metamorphosis in the structure of bacterial communities during the shift from aquatic to terrestrial habits, physiological requirements of adult females involve behavioral and nutritional changes that may also alter their microbiota. Immediately after emergence, adult females predominantly feed on nectar or honeydew to satisfy energetic flight requirements and may introduce diversity and/or favor the proliferation of certain bacteria (Buck et al., 2016). In the present study, *Asaia* which is an acetic acid bacterium could have been horizontally acquired from flower nectar as has already been demonstrated for anopheles mosquitoes (Bassene et al., 2020), or growth could have been enhanced by sugar ingestion, since it was scarce in larvae and newly emerged females, while in wild-caught *An. atroparvus* females it was highly abundant. Moreover, adult

females also ingest blood to fulfill protein requirements for oviposition. Blood digestion produces several changes in internal midgut conditions, which may limit the growth of certain bacteria while enhancing the expansion of others (Wang et al., 2011; Sharma et al., 2020). Accordingly, in *An. atroparvus*, a significant decline in diversity was observed in wild-caught females with the dominance of few bacteria that have been previously reported to succeed during blood digestion, such as *Thorsellia*, *Pantoea*, and *Serratia* (Briones et al., 2008; Wang et al., 2011, 2012; Akorli et al., 2016). Despite the feeding history of wild-caught females in our study being unknown, blood feeding could be evidenced by the gravid status following the inspection of a subset of females from the same cohort (Birnberg et al., 2020). Unexpectedly, *Pseudomonas,* which has been observed to proliferate in the presence of blood (Wang et al., 2011; Sharma et al., 2020) showed an attenuated abundance in wild-caught *An. atroparvus* females, probably blood-fed, a fact that would require further investigation. As evidenced, and consistent with other reports on culicid mosquitoes (Boissiere et al., 2012; Osei-Poku et al., 2012), wild-caught *An. atroparvus* females harbored low diversity but highly variable bacterial communities. This high variation supported the dominant role of the environment in determining the microbiota in adult mosquitoes. Environmentally derived gram-negative bacteria associated with soil, water, plants, and animals dominated the microbiota of *An. atroparvus*, the vast majority from the phylum *Proteobacteria*. Most of the bacterial taxa herein reported have been described as part of the microbiota in culicid mosquitoes (Dada et al., 2014; Muturi et al., 2016; Rocha-David et al., 2016; Hegde et al., 2018; Kang et al., 2020) including *Anopheles* from different geographic regions (Rani et al., 2009; Djadid et al., 2011; Wang et al., 2011; Boissiere et al., 2012; Gimonneau et al., 2014; Ngo et al., 2015; Bogale et al., 2020; Galeano-Castañeda et al., 2020; Zoure et al., 2020; Feng et al., 2021; Silva et al., 2021).

Finally, to achieve an established colony, laboratory breeding constituted a further shift of breeding habitat, which influenced the structure of microbial communities associated with *An. atroparvus*. Contrary to what occurs in the

sylvan environment, the life cycle of the mosquito in the laboratory develops under controlled environmental conditions and is dependent always on the same type of food. Herein, immature stages were maintained in clean dechlorinated tap water and fed an equal amount of balanced fish:turtle food, while adults were offered sterile sucrose and rabbit-blood meals for daily maintenance and oviposition purposes, respectively. It has been suggested that the periodic use of dechlorinated tap water and standard protocols for rearing laboratory colonies have been the cause of diversity loss even among early generations (Akorli et al., 2019; Dada et al., 2020). Conversely, in the transition from wild-caught *An. atroparvus* females to the first laboratory time point analyzed, a significant increase of diversity was observed in laboratory-reared females from the second generation (F2). Interestingly, similar findings were observed only when *Anopheles gambiae* were reared using field-larval water to preserve its field-derived microbiota (Akorli et al., 2019). The bacterial increase in F2 *An. atroparvus* might be linked to a closer relationship with bacteria acquired from their larval breeding habitat, which could have been transiently masked by the dominance of certain taxa acquired and/or proliferated, circumstantially, in wild-caught females due to their physiological needs and/or foraging habits (Buck et al., 2016). This fact could be supported by the similitude of the microbial composition associated with F2 with that of larvae and newly emerged females.

In the following laboratory generations, and consistent with previous studies of other mosquito species (Rani et al., 2009; Coon et al., 2014; Dickson et al., 2018; Akorli et al., 2019), a continuous decline in bacterial diversity was observed in *An. artroparvus* females, although no significant variation was identified up until the 10th generation. This low diversity variation within laboratory colonies may be attributed to standard laboratory conditions and uniform physiological traits in laboratory specimens as previously suggested for *Ae. albopictus* and *An. gambiae* (Minard et al., 2018; Akorli et al., 2019).

Conservation of numerous environmentally acquired bacterial taxa up until the 10th generation, not only suggests the evolutionary conservation of

symbiotic associations of *An. atroparvus* with indigenous bacteria but also evidences the presence of a core microbiota, which may contribute basic information for developing better-adapted vector and disease control strategies. Identifying core symbionts may facilitate the selection of para-transgenesis candidates for interference with pathogen transmission (Wilke and Marrelli, 2015), the generation of axenic/gnotobiotic mosquito models to investigate the effects of the microbiome on mosquito biology without the use of antibiotics (Steven et al., 2021), as well as finding probiotics to improve key factors for population suppression techniques, such as mating performance, mass production, and longevity of sterile males (Chen et al., 2020). In the present study, finding *Serratia* as part of the core microbiota of *An. atroparvus* is promising for local malaria control as *S. marcescens* can reduce mosquito survival, influence the susceptibility of *Anopheles* mosquitoes to *Plasmodium* infections, and decrease parasitical loads (Bando et al., 2013; Bahia et al., 2014; Bai et al., 2019). In fact, *S. liquefaciens* has already been identified as a cultivable bacterium from *An. darlingi* midgut (Arruda et al., 2021), the first step for para-transgenesis. However, it is worth noting that, *S. marcescens* was lost from *An. atroparvus* females during laboratory colonization, a fact that should be further analyzed since it could affect its suitability for para-transgenesis in the studied population. In addition, *Pseudomonas*, identified as the most abundant core genus in *An. atroparvus*, opens up new perspectives for control approaches since it has been suggested as an appropriate candidate for para-transgenesis (Raharimalala et al., 2016), although its role in the biology and vector competence of *An. atroparvus* still needs to be investigated. Furthermore, the high prevalence of *Asaia* in sylvan and laboratory-reared females emphasized its potential use for prevention of malaria in the future and for vector control strategies in Southern Europe. *Asaia* has been proposed as being the most suitable candidate for para-transgenic approaches as it gathers the ecological (e.g., associated with diverse mosquito species; colonizes the midgut, salivary glands, and reproductive organs; horizontally and vertically transmitted),

immunological (e.g., production of anti-plasmodial effector molecules), and technical (e.g., cell-free culture, genetically transformable) requirements for this approach (Favia et al., 2007, 2008; Damiani et al., 2010; Strand, 2017; Rami et al., 2018). Moreover, the high prevalence of *Asaia* in *An. atroparvus* females could explain the absence of *Wolbachia*, as previously described for other *Anopheles* natural populations (Rossi et al., 2015).

To conclude, our study constitutes the first report of the microbiota associated with a sylvan *An. atroparvus* population and significantly contributes to the knowledge of malaria vectors in Europe. Our findings confirm the key role of the breeding environment in shaping the microbiota of vector species and corroborate the decline in diversity during laboratory colonization. The identification of a core microbiota in *An. atroparvus* is a relevant finding that highlights evolutionary conservation of association with its resident bacteria and focuses attention on a limited number for para-transgenic use. Data herein reported may well contribute in creating a well-defined microbiome baseline for further studies on the effects of microbiome manipulation on mosquito phenotypes for malaria research purposes.

Acknowledgments

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Supplementary Material

https://www.frontiersin.org/articles/10.3389/fmicb.2021.775078/full#supplementary-material

Supplementary File 1: OTU Annotation and taxonomic assignations at phylum, family, genus and species levels

Supplementary File 2: Differential abundance analysis at genus level. BaseMean>10, bacteria present in at least 50% of the samples of each group (i.e. W, L, E or Fx) with an average number of normalized sequences higher than 10; padj <0.05, adjusted p-values using FDR correction; log2FC, logarithm 2 of the relative change of each bacterial group.

Supplementary Figure 1: OTU diversity distribution. Donut charts showing the percentage of OTUs annotated at phylum (**A**), family (**B**) and genus (**C**) levels. OTUs identified as "null" represent taxa that were not classified at the given taxonomic level but their classification could be found at lower or higher levels.

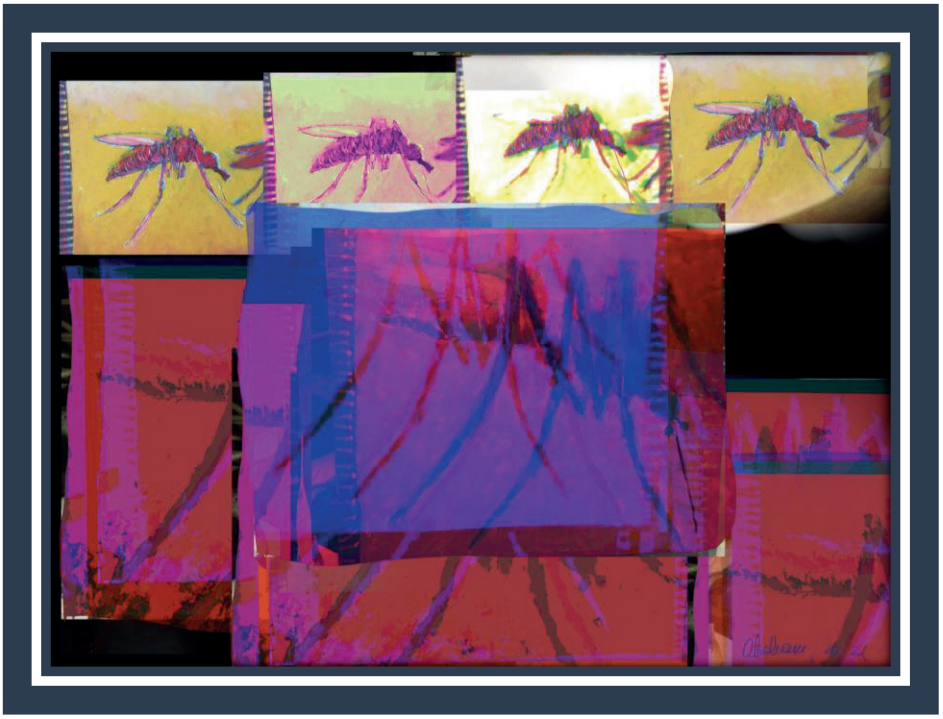
Supplementary Figure 2: Rarefaction curves showing that most of the samples reached the plateau suggesting that the majority of genera were captured at the sequencing depth.

Supplenentary Figure 3: Differences in bacterial community structure. OTU richness and Simpson and Shannon indices estimated at phylum level. Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively. Boxes represent the interquartile range within each group. The line that divides the box corresponds to the median and dots, to minimum and maximum scores. Analysis of variance (ANOVA) significance levels: *p = 0.01–0.05; **p = 0.001–0.01; ***p < 0.001.

Supplenentary Figure 4: Differences in bacterial community structure. OTU richness and Simpson and Shannon indices estimated at genus level. Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively. Boxes represent the interquartile range within each group. The line that divides the box corresponds to the median and dots, to minimum and maximum scores. Analysis of variance (ANOVA) significance levels: *p = 0.01–0.05; **p = 0.001–0.01; ***p < 0.001.

Supplenentary Figure 5: Beta diversity analyses at phylum level depicted microbial community variation. PCoA plot showing bacterial community clustering and segregation according to origin. Color points represent the microbiota of a pool of 20 individuals and color ellipses represent confidence intervals per sample type (**A**). Local contribution to beta diversity analysis (LCBD) showing the uniqueness of bacterial community composition per pool per sample type. The measure of the input is given the size of the black dot (e.g., the larger the dot, the more unique the microbial community) (**B**). Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively.

Supplenentary Figure 6: Beta diversity analyses at family level depicted microbial community variation. PCoA plot showing bacterial community clustering and segregation according to origin. Color points represent the microbiota of a pool of 20 individuals and color ellipses represent confidence intervals per sample type (**A**). Local contribution to beta diversity analysis (LCBD) showing the uniqueness of bacterial community composition per pool per sample type. The measure of the input is given the size of the black dot (e.g., the larger the dot, the more unique the microbial community) (**B**). Sample types: W, breeding water; L, larvae; E, newly emerged females; F0, wild-caught females; F2, F6, and F10, laboratory-reared females from the second, sixth, and 10th generations, respectively.



(Fagment, original illustration: Abraham Birnberg, 2021)

CHAPTER 2

Microbiome and Vector Competence:

Influence of Insect-Specific Flaviviruses on Rift Valley fever phlebovirus Transmission

ASSAY 3

Culex flavivirus infection in a *Culex pipiens* mosquito colony and its effects on vector competence for Rift Valley fever phlebovirus

Talavera, S.; **Birnberg, L.**; Nuñez, A.I.; Muñoz-Muñoz, F.; Vázquez, A. and Busquets, N. *Parasites & Vectors*. (2018), 11:310. doi: 10.1186/s13071-018-2887-4.

ABSTRACT

Rift Valley fever is a mosquito-borne zoonotic disease that affects domestic ruminants and humans. Culex flavivirus is an insect-specific flavivirus that naturally exists in field mosquito populations. The influence of Culex flavivirus on Rift Valley fever phlebovirus (RVFV) vector competence of *Culex pipiens* has not been investigated.

Culex flavivirus infection in a *Cx. pipiens* colony was studied by Culex flavivirus oral feeding and intrathoracical inoculation. Similarly, vector competence of *Cx. pipiens* infected with Culex flavivirus was evaluated for RVFV. Infection, dissemination, transmission rates and transmission efficiency of Culex flavivirus-infected and non-infected *Cx. pipiens* artificially fed with RVFV infected blood were assessed.

Culex flavivirus was able to infect *Cx. pipiens* after intrathoracically inoculation in *Cx. pipiens* mosquitos but not after Culex flavivirus oral feeding. Culex flavivirus did not affect RVFV infection, dissemination and transmission in *Cx. pipiens* mosquitoes. RVFV could be detected from saliva of both the Culex flavivirus-positive and negative *Cx. pipiens* females without significant differences. Moreover, RVFV did not interfere with the Culex flavivirus infection in *Cx. pipiens* mosquitoes.

Culex flavivirus infected and non-infected *Cx. pipiens* transmit RVFV. Culex flavivirus existing in field-collected *Cx. pipiens* populations does not affect their vector competence for RVFV. Culex flavivirus may not be an efficient tool for RVFV control in mosquitoes.

Keywords: Rift Valley fever phlebovirus, *Culex pipiens*, Culex flavivirus, Transmission, Vector competence.

BACKGROUND

Culex flavivirus (CxFV) belongs to the genus *Flavivirus* (family *Flaviviridae*). The majority of viruses within this genus are transmitted horizontally between vertebrate hosts and hematophagous arthropods. However, some flaviviruses are considered to be vertebrate-specific while other group of viruses of this genus are insect-specific (ISFV) (Hoshino et al., 2007; Moureau et al., 2010; Sánchez-Seco et al., 2010). Circulation of ISFVs in natural mosquito populations is likely maintained by vertical transmission (Sang et al., 2003; Lutomiah et al., 2007). In Europe, several species of ISFV have been detected in field mosquitoes from Italy, Portugal, Spain, the United Kingdom, the Czech Republic and Greece (Calzolari et al., 2012; Cerutti et al., 2012; Vazquez et al., 2012; Osório et al., 2014; Papa et al., 2014). Sequences related to those viruses have been detected worldwide (Ochieng et al., 2007; Pabbaraju et al., 2009; Hoshino et al., 2012; Datta et al., 2015). ISFV RNA has also been detected in sand flies (family *Psychodidae*) in Algeria (Moureau et al., 2010), Spain (Sánchez-Seco et al., 2010) and Portugal (GenBank: HM563684). Previous field studies in Spain suggested the existence of a large number of ISFV (Aranda et al., 2009; Sánchez-Seco et al., 2010; Alba et al., 2014), though not completely characterized phylogenetically (Vazquez et al., 2012). The circulation of ISFV in nature raises concerns regarding possible interactions with arthropod-borne flaviviruses (Crabtree et al., 2003) and even other arboviruses in vector populations. Co-infection studies with mosquito-borne flaviviruses (MBFV) and ISFV have been performed in order to gain a better understanding of any factor that could alter vector competence of mosquitoes for MBFV in both enzootic and epizootic transmission cycles (Goenaga et al., 2015). Three studies were carried out to directly address potential co-infection exclusion effect between CxFV and other flaviviruses such as West Nile virus (WNV) (Kent et al., 2010; Bolling et al., 2012; Goenaga et al., 2015). However, no co-infection studies with other pathogenic viruses belonging to other genera have been performed, such as Rift Valley fever phlebovirus (RVFV).

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease caused by RVFV (genus *Phlebovirus*, family *Phenuiviridae*). RVFV is transmitted by mosquito bites to a large number of hosts, both domestic and wild ruminants (Olive et al., 2012). Described for the first time in 1931 in Kenya (Daubney et al., 1931), RVFV has continuously caused outbreaks in animals and humans in several African countries (Nanying et al., 2015). In 2000, RVFV was first reported outside of Africa, i.e. in Saudi Arabia and Yemen (Ahmad, 2000), linking to the likelihood of a potential introduction of RVFV in Europe. The risk of RVFV introduction in Europe has been recently evaluated (Chevalier et al., 2010; Rolin et al., 2013; Sánchez-Vizcaino et al., 2013; Mansfield et al., 2015). Results of a multiple criteria decision-making model study of key factors for RVF in Spain identified areas with high suitability for RVF outbreak occurrence in each month of the year (Sánchez-Vizcaino et al., 2013). Moreover, a previous study has shown that a *Culex pipiens* mosquito colony from Spain is able to transmit this virus (Brustolin et al., 2017). Species of the genera *Aedes* and *Culex* are considered main vectors of RVFV (Abdo-Salem et al., 2012). *Culex pipiens* complex is considered as an efficient RVFV vector (Turell et al., 1996) including *Cx. pipiens* and *Cx. quinquefasciatus*, which are ubiquitous mosquitoes in temperate and tropical regions, respectively (Amraoui et al., 2012).

It is relevant to understand ISFV dynamics and their role in their mosquito hosts as potential control tool for vector-borne pathogens. To this end, the objectives of the present study were to evaluate (i) the CxFV infection in a *Cx. pipiens* colony by oral feeding and intrathoracic inoculation and (ii) the role in vector competence of CxFV for RVFV infection, dissemination and transmission by *Cx. pipiens*. All experiments were performed simulating environmental conditions of the season with high vector density and high suitability for RVF outbreak occurrence in the distribution area of the tested mosquito population.

MATERIALS AND METHODS

Mosquito populations

One mosquito population of *Cx. pipiens pipiens* and *molestus* hybrid form from Gavà (2012), Catalonia (northeastern Spain) was used. Molecular characterization of the *Cx. pipiens* forms was performed for each individual involved in the RVFV vector competence assay as previously described (Bahnck and Fonseca, 2006). The *Cx. pipiens* colony was reared in laboratory under environmental conditions: temperature, 26 °C:22 °C (day:night); relative humidity (RH) of 80%; and a 14:10 h (L:D) photoperiod including two crepuscular cycles of 30 min to simulate dawn and dusk.

Before vector competence assays, the mosquito colony was tested for the presence of viruses, as described previously (Brustolin et al., 2017), to exclude other viral infections (species of *Flavivirus*, *Alphavirus* and *Phlebovirus*). In the last decade, other novel insect-specific viruses have been detected in field mosquitoes belonging to several families such us *Bunyaviridae*, *Mesoniviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae* and the newly recognized taxon of *Negeviruses* (Vasilakis and Tesh, 2015). Prior to vector competence assays, the colony was also tested for the presence of these viruses using generic RT-nested-PCR (unpublished) and *Wolbachia* spp. by PCR (Zhou et al., 1998). The mosquito colony was found to be *Wolbachia* spp.-positive and negative for *Flavivirus*, *Alphavirus*, *Phlebovirus*, *Bunyaviridae*, *Mesoniviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae* and *Negeviruses* (data not shown).

Virus strains

The CxFV strain was detected in field-collected *Culex pipiens* mosquitoes captured in Huelva, Spain, in 2006, and isolated in C6/36 cells. To propagate the virus, C6/36 cells were incubated for 6–7 days (28 °C, 5% CO_2) and viral particles were observed by electronic microscopy. As cytopathic effect was not observed, CxFV replication was detected in the supernatant using a modified

real time RT-PCR (Bolling et al., 2012) (see below). A monolayer of C6/36 cells was used to titrate CxFV. Briefly, eight wells were infected for each ten-fold dilution. Twenty microliters of inoculum and 150 µl of minimum essential medium (Life Technologies, Carlsbad, CA, USA) supplemented with 2% FBS (EuroClone SpA, Pero, Italy), 2 mM L-glutamine, nonessential amino acids, 1000 U/ml of penicillin, 10 mg/ml of streptomycin and 500 U/ml of nystatin (all from Sigma-Aldrich, St. Louis, MO, USA), were added into each well as post-infection medium. Plates were incubated at 28 °C and 5% of CO_2 for 7 days. Calculation of the viral titer was performed by virus detection in each well using real time RT-PCR. Ct-values ranged between 21.09–23.48 in the wells where the virus replicated. The 50% tissue culture infective dose per milliliter ($TCID_{50}$/ml) was calculated using the Reed & Muench method (Villegas, 1980).

The virulent RVFV 56/74 strain (passages history (Busquets et al., 2010) and one passage in C6/36 cells) was propagated in BHK-21 cells. The virus was titrated in Vero cells and cytopathic effect was observed. The 50% tissue culture infective dose per milliliter ($TCID_{50}$/ml) was also calculated using the Reed & Muench method (Villegas. 1998).

CxFV infection in mosquitoes orally exposed

Fourteen-day-old *Cx. pipiens* females were exposed for 60 min to CxFV infected blood (1:2) at 4 $\log_{10}$ $TCID_{50}$/ml using the Hemotek feeder system. At 0, 3, 5, 7 and 10 days post-exposure (dpe), six fed females were harvested and frozen until analysis.

CxFV intrathoracic inoculation in mosquitoes

A group of 36 *Cx. pipiens* females, 2–3 days of age, were intrathoracically inoculated with CxFV at 4 $\log_{10}TCID_{50}$/ml diluted 1:2 in Dulbecco's modified Eagle medium (DMEM). To study virus replication kinetics, these females were examined at 0, 3, 5, 7, 9 and 11 days postinoculation (dpi). Bodies were

analyzed from the 36 mosquitoes and saliva was harvested from all mosquitoes except from those corresponding to 0 dpi. Saliva was collected using a capillary technique as previously described (Brustolin et al., 2017). As an inoculation control, a group of mosquitoes was inoculated with only DMEM.

RVFV vector competence assay

The ability of RVFV to infect, disseminate and be transmitted by *Cx. pipiens* infected and non-infected with CxFV was evaluated by: infection rate (IR), disseminated infection rate (DIR), transmission rate (TR) and transmission efficiency (TE). IR refers to the proportion of mosquitoes with infected body among tested mosquitoes. DIR corresponds to the proportion of mosquitoes with infected legs/wings among the previously detected infected mosquitoes (i.e. body positive). TR represents the proportion of mosquitoes with infected saliva among mosquitoes with disseminated infection. TE represents the proportion of mosquitoes with infected saliva among the total number of mosquitoes tested (Chouin-Carneiro et al., 2016).

Seven- to nine-day-old female mosquitoes that had never been blood-fed were used. Mosquitoes were reared and fed as previously described (Brustolin et al., 2017). *Culex pipiens* intrathoracically inoculated with CxFV or with DMEM were tested for vector competence (VC) using a RVFV viral dose of 7.23 $\log_{10}TCID_{50}$/ml. After the blood-feeding, CO_2 was used to anesthetize the mosquitoes and fully engorged females (FEF) were selected. The blood doped with RVFV was titrated in Vero cells. Ten percent of the specimens from each group were sacrificed and analyzed as a control of the inoculum. The rest of the mosquitoes were individually placed to cardboard cages (Watkins & Doncaster, Leominster, UK).

FEF were fed with sucrose (10%) *ad libitum* using soaked cotton pledgets. The presence of viral RNA in saliva was evaluated using two different approaches: FTA™ cards (GE Healthcare, Little Chalfont, UK) soaked with Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zealand) and

a blue alimentary colorant, at 4 and 14 dpe and the direct extraction of mosquitoes' saliva by capillarity at 14 dpe. At 4 and 14 dpe the FTA cards were left 24 h on the top of the mesh screen of all cardboard cages to allow the mosquito to feed on it. After FTA cards collection, they were resuspended in 0.3 ml of PBS and stored at -80 °C until tested. At 14 dpe, every mosquito was anesthetized with CO_2 and dissected, and samples (legs/wings and bodies) were collected as previously described (Brustolin et al., 2017). One hundred-fifty microliters from the saliva sample contained in DMEM medium were used for viral RNA extraction and the remaining 50 µl were used for RVFV isolation in a Vero cells monolayer. Cells were incubated for 7 days (37 °C, 5% CO_2) and the cytophatic effect was evaluated.

Virus detection

CxFV detection was performed using the real time RT-PCR protocol described by Bolling et al., 2012 with minor modifications. The primer CxFV-F was modified as follows: 5'-CTA CGC TCT TAA CAC AGT GA-3' and RT-qPCR was carried out using Quantitec SyBr Green RT-PCR kit (Qiagen, Hilden, Germany). Samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) programmed as follows: 50 °C for 10 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s and at 57 °C for 35 s. RVFV RNA was extracted and detected as previously described (Brustolin et al., 2017).

Statistical analysis

The frequency with which CxFV (+) and CxFV (-) mosquitoes get infected, disseminate, and transmit RVFV was compared by Fisher's exact test. Ct-values in mosquito bodies, legs/wings and saliva 14 dpe were compared between CxFV (+) and CxFV (-) groups by a non-parametric Mann-Whitney test as data were not normally distributed. Differences in Ct-values in CxFV inoculated mosquitoes among dpi were assessed by means of a multiple

comparisons Kruskal-Wallis test. *P*-values < 0.05 were considered statistically significant.

RESULTS

CxFV replication kinetics in orally exposed *Cx. pipiens*

No CxFV replication was detected in *Cx. pipiens* exposed orally, suggesting that *Cx. pipiens* mosquitoes are not susceptible to CxFV infection by oral exposure. Although no positive CxFV was recorded in any tested female mosquito on 3, 5, 7 and 10 dpe, CxFV could be detected in all mosquito samples collected on 0 dpe, demonstrating that all mosquitoes were exposed to the virus (**Figure 1**).

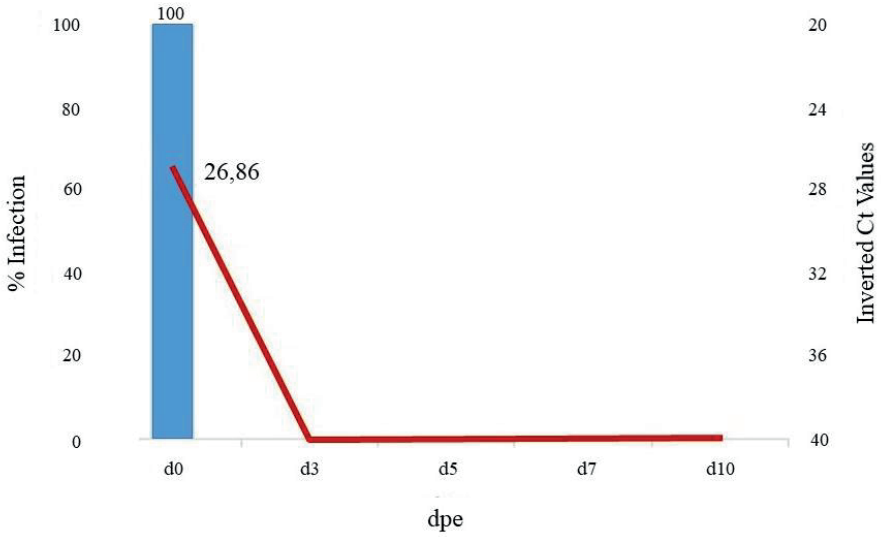


Figure 1. CxFV replication kinetics in *Cx. pipiens* oral infection. *Cx. pipiens* mosquitoes were not susceptible to CxFV infection following oral exposure. Columns show infection percentages and the line represents the Ct-values obtained by RT-qPCR. *Abbreviation*: dpe, days post-exposure.

CxFV replication kinetics in *Cx. pipiens* intrathoracically inoculated

Culex pipiens intrathoracically inoculated with CxFV showed viral replication. Results demonstrated a high percentage of CxFV infection detected at all time-points analyzed. The obtained Ct-values were high, indicating low viral load. However, the multiple comparison Kruskall-Wallis test detected significant differences in viral loads among dpi (H = 16.692, df = 5, P = 0.005). The multiple comparisons of mean ranks indicated that the viral load in bodies of females tested at 7 and 9 dpi was significantly higher than at 0 dpi (z = 3.33, P = 0.012 and z = 3.06, P = 0.033, respectively), showing CxFV replication within *Cx. pipiens* after intrathoracic inoculation (**Figure 2**). All saliva samples tested at different time points were negative to CxFV.

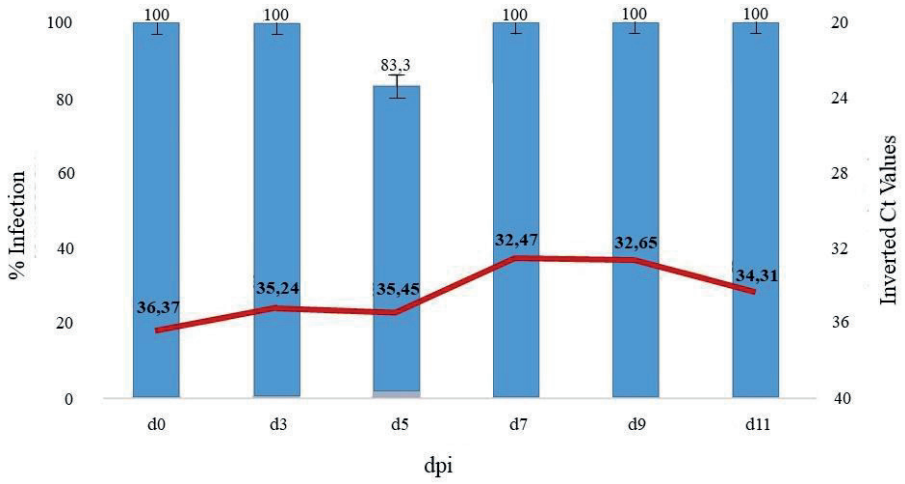


Figure 2. CxFV replication kinetics in *Cx. pipiens* intrathoracilally inoculated. *Cx. pipiens* mosquitoes were susceptible to CxFV infection after intrathoracic inoculation. Columns show infection percentages and the line represents the Ct-values obtained by RT-qPCR. *Abbreviation*: dpi, days post-inoculation.

CxFV replication kinetics in *Cx. pipiens* co-infected with RVFV

CxFV replication was not affected by RVFV exposure in female *Cx. pipiens* mosquitoes. Results showed that 21 days after CxFV inoculation and 14 days after RVFV exposure (14 dpe), bodies of all tested females remained positive to CxFV without significant differences (**Figure 3**).

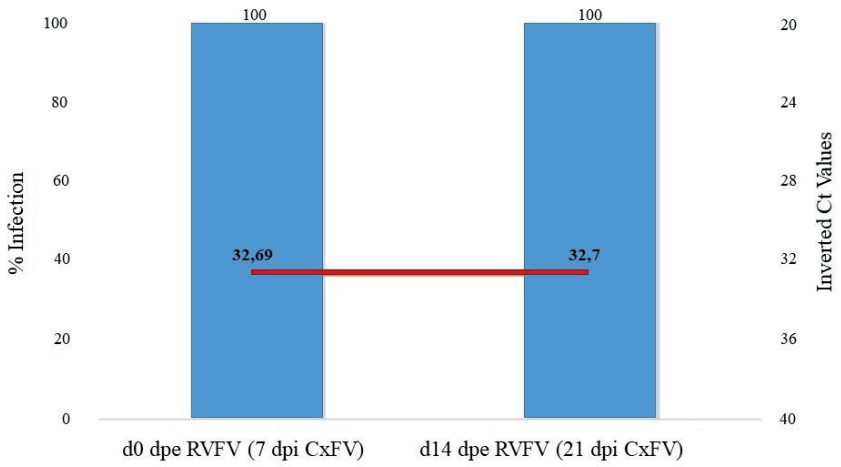


Figure 3. CxFV replication kinetics in co-infection with RVFV in *Cx. pipiens*. CxFV persisted after 21 dpi and was not influenced by RVFV exposure. Columns show infection percentages and the line represents the Ct-values obtained by RT-qPCR.

RVFV infection, dissemination and transmission in *Cx. pipiens* infected and non-infected with CxFV

Mosquitoes infected with CxFV and exposed to RVFV (n = 10; n = 1 hybrid form and n = 9 molestus form) and mosquitoes non-infected with CxFV and exposed to RVFV (n = 22; n = 5 hybrid form and n = 17 molestus form) were analyzed at 14 dpe. The percentages of RVFV infection, dissemination and transmission in analyzed mosquito females were not significantly different between females infected and non-infected with CxFV (**Table 1**). Moreover, RVFV loads in bodies and legs/wings were not significantly different between females infected and non-infected with CxFV (**Figure 4**).

Table 1. RVFV infection, dissemination and transmission in *Cx. pipiens* infected and non-infected with CxFV

CxFV infection	IR	DR	TR	TE
+	5/10 (50%)	2/5 (40%)	1/2 (50%)	1/10 (10%)
-	15/22 (68%)	5/15 (33%)	4/5 (80%)	4/22 (18%)

Notes: IR, infection rate; DR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency

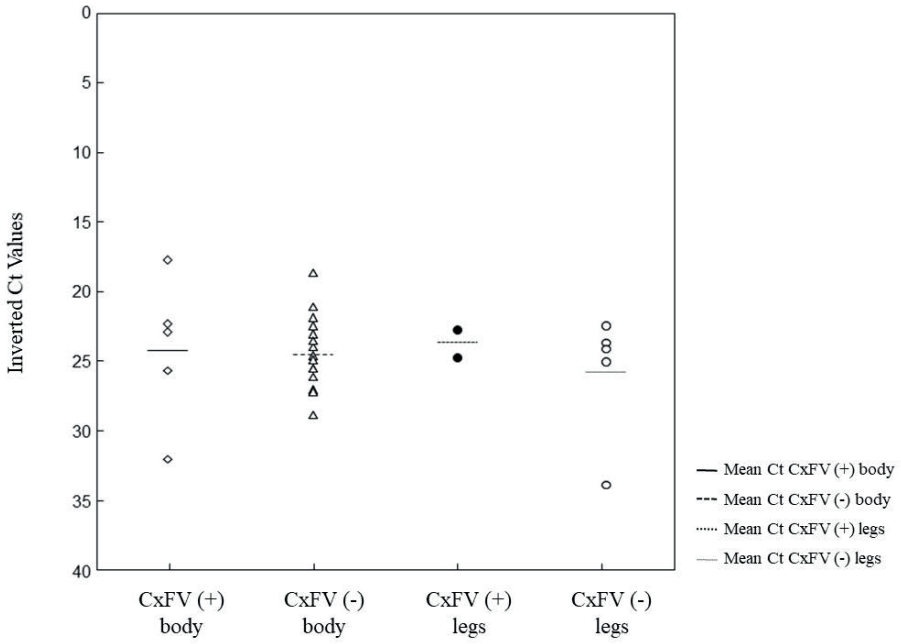


Figure 4. RVFV Ct-values in female mosquito bodies and legs infected and non-infected with CxFV. RVFV loads in female mosquito bodies and legs/wings were not affected by CxFV infection.

All RVFV-positive saliva were detected in females with disseminated infection at 14 dpe. The Ct-values in mosquito saliva did not differ significantly between both groups, infected and non-infected with CxFV (**Table 2**). In

addition, RVFV was detected in bodies, legs/wings or saliva of mosquitoes with (n = 27) and without (n = 5) *Wolbachia* (**Table 2**).

Table 2. Presence of RVFV in different samples of mosquitoes with positive saliva at 14 dpe. Ct-values of positive samples analyzed by RT-qPCR are reported

Individuals	**Legs and Wings**	**Saliva**	**Saliva (CPE)**	**CxFV**	***Wolbachia***
Cx. pipiens molestus	22,76	32,40	-	29,49	-
Cx. pipiens molestus	22,43	30,55	-	-	+
Cx. pipiens molestus	23,70	34,13	-	-	+
Cx. pipiens molestus	24,10	32,54	-	-	+
Cx. pipiens molestus	25,00	38.39	-	-	+

Abbreviations: -, negative; +, positive; CPE cytopathic effect

Regarding the forms of individuals from the *Cx. pipiens* hybrid colony, RVFV was detected in mosquito bodies, legs/wings and saliva of *Cx. pipiens* form molestus and in mosquito bodies of the hybrid form (**Table 3**).

Table 3. RVFV infection, dissemination and transmission in *Cx. pipiens* molestus form individuals, hybrid form individuals and all individuals of total mosquitoes tested

Cx. pipiens	**IR**	**DR**	**TR**	**TE**
Molestus form (individuals)	16/26 (61%)	7/16 (44%)	5/7 (71%)	5/26 (19%)
Hybrid form (individuals)	4/6 (67%)	0/4 (0%)	-	0/6 (0%)
Total (colony)	20/32 (62%)	7/20 (35%)	5/7 (71%)	5/32 (16%)

Abbreviations: IR infection rate, DR disseminated infection rate, TR transmission rate, TE transmission efficiency.

DISCUSSION

The isolation, identification and characterization of numerous insect-specific viruses in recent years are of particular interest. They can coexist with pathogenic arboviruses in mosquito populations and may potentially affect the transmission of vector-borne infectious diseases. While there is extensive genetic and phenotypic characterization of insect-specific flaviviruses, little is known about the interactions between them and their mosquito hosts and other arboviruses and the potential public health significance of these associations (Crockett et al., 2012). Relatively few studies have been performed on co-infections with other flaviviruses such as WNV (Kent et al., 2010; Bolling et al., 2012; Goenaga et al., 2015). To the best of our knowledge, the present study is the first to perform a co-infection with two viruses from different genera, CxFV (*Flavivirus*) and RVFV (*Phlebovirus*).

The mechanism through which natural mosquito populations become infected with CxFV is not yet well defined. Our results strongly suggest that *Cx. pipiens* females are not susceptible to CxFV upon oral exposure. This is in agreement with previous studies showing transmission of insect-specific viruses solely among their invertebrate hosts by vertical route (Sang et al., 2003; Hoshino et al., 2007). Intrathoracic inoculation of CxFV in our study, however, indicates that the virus may have the potential to replicate in *Cx. pipiens* females at least for 21 days, establishing a possible CxFV persistent infection. Nevertheless, CxFV could not be detected in saliva after 14 dpi. Our results are in line with a previous report by Kent et al., 2010 who showed that CxFV Izabal intrathoracically inoculated to *Cx. quinquefasciatus* females was not found in the saliva.

Vector competence for RVFV was examined at 14 dpe in one *Cx. pipiens* colony artificially infected with CxFV by intrathoracic inoculation. The percentage of mosquito females that became infected, developed a disseminated infection, and transmitted RVFV was not significantly different between females infected and non-infected with CxFV. We assume that CxFV

may have co-evolved with their mosquito host evading their immune system without affecting its function against a subsequently-infecting virus. As such, the molecular mechanisms that allow co-existence of both CxFV and RVFV are not well defined and need more extensive studies. Furthermore, RVFV RNA levels observed were also not significantly different suggesting that CxFV does not affect RVFV replication. This is in agreement with other published studies where co-infection of CxFV and WNV has been performed. Similarly, Kent et al., 2010 investigated the vector competence for WNV of *Cx. quinquefasciatus* mosquitoes intrathoracically inoculated with CxFV Izabal, and also observed no significant differences in WNV titers between CxFV-positive and CxFV-negative mosquitoes at 14 dpi. Another study that tested the vector competence for WNV in two *Cx. pipiens* colonies (Bolling et al., 2012), one colony CxFV naturally infected and the other CxFV non-infected, reported no significant differences in WNV dissemination between both colonies at 14 dpe. However, significant differences were observed at 7 dpe, being significantly higher in the CxFV-negative colony than in CxFV-positive colony. These results suggested a competitive interaction between CxFV and WNV indicating a possible early suppression of WNV replication by CxFV infection in *Cx. pipiens*. Vector competence is influenced by the time-point examined and by genetic differences between mosquito populations (Bennett et al., 2002) as well as genetic diversity and fitness of a laboratory-colonized population (Lorenz et al., 1984; Lambrechts et al., 2010). All these factors must be taken into account for co-infection studies in mosquitoes.

The *Cx. pipiens* colony used in the present study was naturally infected by *Wolbachia* spp. This may have influenced the vector competence of infected mosquitoes as shown in a previous study (Moreira et al., 2009). Our results showed that RVFV was detected in bodies, legs/wings or saliva of mosquitoes with (n = 27) and without (n = 5) *Wolbachia*. Due to the small sample size, further studies regarding this issue are needed to explain the potential interference of *Wolbachia* in arbovirus-vector interactions.

The present study and our previous report (Brustolin et al., 2017) allow us to assure that the *Cx. pipiens* hybrid colony of Gavà can become infected, disseminate and transmit RVFV. The IR and DIR obtained were lower than those reported by Turrell et al., 2014 when a *Cx. pipiens* hybrid colony was exposed to a similar RVFV viral dose (107.5 PFU/ml) at 14 dpe. Regarding the forms of *Culex pipiens*, RVFV was detected in mosquito bodies, legs/wings and saliva of *Cx. pipiens* form molestus (n = 26 tested). Thus, our findings in the present work also showed that the individuals of molestus form within the hybrid colony disseminated and transmitted RVFV. However, the virus was only detected in mosquito bodies in hybrid form (n = 6). These results may suggest that the individual form might determine the RVFV dissemination and later transmission, suggesting a strong midgut barrier in hybrid form in *Cx. pipiens* individuals.

The insect's immune responses largely determine the viral load, extrinsic incubation period, and mortality of the insect vector after viral infection, all of which directly affect the outcome of disease transmission (Ocampo et al., 2013; Sim et al., 2014). Exposure to one microorganism can provide cross-protection against another microorganism. Specific examples of the super-infection exclusion hypothesis based on the idea of homologous interference, which is the ability of an established infection with one virus to interfere with secondary viral infection, has been documented in cell culture not only with flaviviruses (Sundin and Beaty, 1988; Randolph and Hardy, 1998; Burivong et al., 2004; Pepin et al., 2008), but also with other arboviruses of the genera *Alphavirus* (Karpf et al., 1997), *Orbivirus* (Ramig et al., 1989) and *Vesiculovirus* (Legault et al., 1977; Whitaker-Dowling et al., 1983). The study of Bolling et al., 2012 reported that CxFV could alter the WNV infection on mosquitoes although it did not exclude WNV infection. However, a positive correlation between WNV and CxFV infection of field-collected *Cx. pipiens* mosquitoes from Illinois has been observed, suggesting that there could be a biological suppression that mediates an increasing susceptibility to naturally WNV infected mosquitoes (Newman

et al., 2011). Moreover, WNV transmission was enhanced in the Honduras colony when mosquitoes were inoculated simultaneously with WNV and CxFV Izabal (Kent et al., 2010). To our knowledge, nothing was known about the potential interference of CxFV in the mosquito infection by other arboviruses not belonging to *Flavivirus* genus. Our results, for the first time, indicate that CxFV infection in *Cx. pipiens* might not alter the immune system to interfere with the RVFV infection in case of RVFV introduction in *Cx. pipiens* populations.

CONCLUSIONS

This is the first study to assess the potential interference of an ISF on RVFV transmission. We have shown that CxFV does not affect RVFV infection, dissemination and transmission. Mosquitoes persistently infected at the assessed conditions may not be used as a preventive intervention strategy for blocking the transmission of RVFV. Further studies using mosquitoes naturally infected with CxFV should be performed to deepen the knowledge in the natural CxFV infection and to elucidate consistent trends for RVFV vector competence in CxFV artificially and naturally infected *Cx. pipiens* populations. Altogether, it is necessary to highlight the importance of deepening the knowledge on the interaction of ISF circulating in mosquito populations present in an area where the potential pathogenic arboviruses can be introduced in order to better assess arbovirus risk transmission. Examining associations between insect-specific viruses such as CxFV and RVFV and other arboviruses important for human and animal health will provide significant new insights into both arbovirus biology and public health.

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ASSAY 4

Field-captured *Aedes vexans* (Meigen, 1830) is a competent vector for Rift Valley fever phlebovirus in Europe

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ABSTRACT

Aedes vexans (Meigen) is considered a nuisance species in central Europe and the Mediterranean region. It is an anthropophilic and mammalophilic floodwater mosquito involved in the transmission of several arboviruses. Rift Valley fever (RVF) is a relevant mosquito-borne zoonosis, affecting mainly humans and ruminants, that causes severe impact in public health and economic loses. Due to globalization and climate change, the European continent is threatened by its introduction. The main purpose of the present study was to evaluate the vector competence of a European field-collected *Ae. vexans* population.

Aedes vexans field-collected larvae were reared in the laboratory under field-simulated conditions. To assess the vector competence for Rift Valley fever phlebovirus (RVFV) transmission, adult F0 females were exposed to infectious blood meals containing the 56/74 RVFV strain. Additionally, intrathoracic inoculations with the same virus strain were performed to evaluate the relevance of the salivary gland barriers. Natural circulation of alphavirus, flavivirus and phlebovirus was also tested.

To our knowledge, an autochthonous *Ae. vexans* population was experimentally confirmed as a competent vector for RVFV for the first time. This virus was capable of infecting and disseminating within the studied *Ae. vexans* mosquitoes. Moreover, infectious virus was isolated from the saliva of disseminated specimens, showing their capacity to transmit the virus. Additionally, a natural infection with a circulating Mosquito flavivirus was detected. The co-infection with the Mosquito flavivirus seemed to modulate RVFV infection susceptibility in field-collected *Ae. vexans*, but further studies are needed to confirm its potential interference in RVFV transmission.

Our results show that field-collected European *Ae. vexans* would be able to transmit RVFV in case of introduction into the continent. This should be taken into consideration in the design of surveillance and control programmes.

Keywords: *Aedes vexans*, RVFV, Mosquito Flavivirus, Vector competence

BACKGROUND

Aedes vexans (Meigen, 1830) is a floodwater mosquito widely distributed throughout the Holarctic region and it is native in Eastern Europe. This species inhabits a variety of habitats, especially within rural areas (Becker et al., 2003). It mostly breeds in floodplains, rivers and lakes. As most floodwater mosquitoes, *Ae. vexans* lay their eggs near temporary or semi-permanent ground pools predisposed to seasonal inundations. Their eggs in diapause survive long periods of drought and hatch massively after flooding episodes. *Aedes vexans* is able to complete its developmental cycle in only a few days producing high population densities (Miller et al., 2002). Adult females are aggressive bitters with low host specificity among mammals and humans (Börstler et al., 2016), relevant for potential pathogen transmission. In North America and Europe, several arboviruses, such as West Nile virus (WNV), Snowshoe hare virus (SSHV), Jamestone Canyon virus (JCV) (Anderson et al., 2015), Tahyna virus (TAHV) (Gligić and Adamović, 1976), and Batai virus (BATV) (Scheuch et al., 2018) to name a few, have been isolated from *Ae. vexans*. In Africa, *Ae. vexans* is considered one of the primary vectors of Rift Valley fever phlebovirus (RVFV) (Talla et al., 2016; Sang et al., 2017), and has been found naturally infected with the virus (Ndiaye et al., 2016). In addition, its competence in the transmission of RVFV has been confirmed experimentally in field populations from Africa and the USA (Turell et al., 2008, 2013; Ndiaye et al., 2016).

Rift Valley fever (RVF) is a zoonotic vector-borne viral disease that mainly affects domesticated ruminants and humans. Rift Valley fever is responsible for high mortality rates in newborn and juvenile ruminants, and abortions in pregnant animals (Chevalier et al., 2010). Human infections may vary from an asymptomatic to mild febrile illness, but 1% of them may develop into severe encephalitis, haemorrhagic fever and death (Mansfield et al., 2015). Its causal agent, RVFV, belongs to the genus *Phlebovirus* within the family *Phenuiviridae*. Unlike most phleboviruses, which are primarily transmitted by

sand flies, RVFV is transmitted predominantly by infected mosquito bites (Turell et al., 2008).

Due to its dreadful impacts on public health and the economy in endemic countries, RVFV belongs to the World Organization for Animal Health (OIE)'s list of notifiable animal diseases of concern, and is classified as a category A priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) (Hartman, 2017). In the last decades, RVFV distribution has expanded from its original location in sub-Saharan Africa to North and West Africa, the Arabian Peninsula, Mayotte Island and Madagascar (Chevalier et al., 2010; Mansfield et al., 2015; Ndiaye et al., 2016; Samy et al., 2017). Although no RVF cases have been reported in Europe so far, globalization and climate change have raised concerns of its introduction through the Mediterranean basin. While predictive risk models of the introduction of RVF within the European Union have reported a low risk (Chevalier et al., 2010), a study using a spatial multicriteria decision-making (MCDM) model for RVF outbreak occurrence in Spain, showed a high suitability for RVF in the east-coast regions (Sánchez-Vizcaino et al., 2013), where *Ae. vexans* mosquito is present.

For a better understanding of the potential role in the transmission of RVFV of an autochthonous population of *Ae. vexans* in Europe, we tested the ability of field-captured *Ae. vexans* mosquitoes from Begues municipality in Catalonia (Spain) for the transmission of RVFV.

MATERIALS AND METHODS

Sample collection and mosquito rearing

In September 2016 and May 2019, after heavy rain episodes, *Ae. vexans* third- and fourth-instar larvae were collected from Begues municipality (41°19′57.94″N, 1°54′20.40″E) (Catalonia, Spain). To obtain an F0 generation, larvae were reared in the laboratory under local field-simulated conditions

(photoperiod 14 h day:10 h night, relative humidity: 80%, temperature: 22–26 °C) using the same water and substrate from their original breeding site. Specimen identification was based on morphology as described by Schaffner et al., 2001.

Virus strain and inoculum preparation

A South African virulent 56/74 RVFV strain (viral stock provided by Alejandro Brun, INIA), isolated from cattle in 1974 (Barnard and Botha, 1977) was used. The virus was passaged twice in *Aedes albopictus* clone C6/36 cells and titrated in African green monkey kidney (Vero) cells (both cell lines provided by Joan Pujols, IRTA-CReSA, Barcelona, Spain) to obtain a 50% tissue culture infective dose per milliliter ($TCID_{50}$/ml) (Busquets et al., 2010). For mosquito blood meals, fresh heparinized bovine blood (*Servei de granja i camps experimentals* (SGICE), Veterinary Faculty, Autonomous University of Barcelona) was supplemented with adenosine 5'-triphosphate (ATP) disodium salt hydrate (5 × 10−3 M) (Sigma-Aldrich, St. Louis, MO, USA) as phagostimulant. Infectious blood meals were prepared by mixing (1:3) bovine blood and virus to obtain a final concentration of 7.5 $\log_{10}$ $TCID_{50}$/ml. The viral dose employed in our assay was similar to those detected previously in blood samples from experimentally infected European lambs (Busquets et al., 2010).

Design of the vector competence assay

The competence for the transmission of RVFV of a European field-captured *Ae. vexans* population was assessed in two different years, 2016 and 2019. In 2016, at the *Institut de Recerca i Tecnologies Agroalimentaries – Centre de Recerca en Sanitat Animal* (IRTA – CReSA) Biosafety Level 3 (BSL3) facilities, 422 non-blood-fed F0 females aged 7–9 days were exposed to artificial feedings. All F0 females were starved for 24 h and exposed to an infectious blood meal that was performed using a Hemotek feeding system (Discovery Workshop, Accrington, UK) set at 37.5 °C for one hour. A specific pathogen-free (SPF) chicken skin served as a feeding membrane.

Simultaneously, a virus-free blood meal was offered to one group to obtain a negative control. After feedings, specimens were anesthetized by exposing them to carbon dioxide (CO_2); fully engorged females (FEF) were separated and kept in individual cardboard cages (Watkins & Doncaster, Leominster, UK) under rearing conditions (photoperiod 14 h day:10 h night, relative humidity: 80%, temperature: 22–26 °C). On the same day of feeding, three FEF from each group were sacrificed to verify the presence or absence of the virus. The remaining unfed females were maintained deprived of sucrose for another 24 h and subjected to a second feeding (following 48 h of starvation). The same procedure for feeding and female classification were performed, with the only difference to verify the infectious status, five FEF were sacrificed per group. Twenty-one (17 exposed to RVFV and four from the negative control) and 40 FEF (35 exposed to RVFV and five from the negative control), from the first and second feeding, respectively, were maintained for 14 days under rearing conditions until the completion of the extrinsic incubation period (EIP).

At 14 dpe, all specimens were anesthetized with CO_2. Legs and wings were removed from the body of each specimen and stored in 1.5 ml tubes containing 0.5 ml Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland). Immediately after dissection, saliva samples were collected by the capillary technique used by Brustolin et al. 2017. All samples were stored at − 80 °C until processed. Specimens from the negative control group helped to verify the survival of the studied individuals and their infection status until the end of the experiment.

In 2019, 229 F0 females were obtained from field-collected larvae. Prior artificial feeding, 148 7–9 day-old non-blood-fed females were deprived of sucrose for 48 h to ensure a higher feeding rate. Artificial feeding, specimen maintenance, sample collection and processing were performed as described above for the previous assay.

Since the number of disseminated specimens after oral exposure to RVFV was low, to better evaluate the transmission rate of this mosquito population, as well as, to assess the relevance of the salivary glands barriers, intrathoracic inoculations were performed.

RVFV intrathoracic inoculation in mosquitoes

Using a XenoWorks analog microinjector (BRI) (Sutter Instrument, CA, USA), 67 9–12 day-old females, from the same 2019 batch, were inoculated with 1–2 µl of the same RVFV strain (5.67 $\log_{10}TCID_{50}$) previously used in artificial feeding assays. Fourteen specimens were inoculated with sterile PBS as an inoculation and survival control. To confirm the infection status, five specimens were sacrificed the same day of microinjection. Inoculated specimens were maintained individually for 7 days under previous rearing conditions. At day 7 post-inoculation (7 dpi) all specimens were anesthetized with CO_2, legs and wings were detached from the body, and the saliva of all females harvested as previously described for artificial feeding. Bodies, legs and wings, and saliva samples were stored at − 80 °C until molecular analysis could be completed.

Detection and isolation of RVFV

Viral RNA was extracted from bodies, legs and wings, and saliva samples using NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany). RVFV detection and quantification were performed following the protocol previously described Brustolin et al., 2017 where the limit of detection was established at 0.09 $TCID_{50}$ per reaction. Quantification cycle (Cq) values below 36 were considered positive for RVFV. Saliva samples were also incubated in Vero cells (37 °C, 5% CO_2) for RVFV isolation for 7 days, before cytopathic effect was visually evaluated.

Parameters to evaluate *Ae. vexans* vector competence for RVFV

At 14 dpe, infection, disseminated infection and transmission rates (IR, DIR and TR, respectively), and transmission efficiency (TE) were estimated. IR

corresponds to the fraction of FEF whose bodies tested positive for RVFV. DIR is the proportion of FEF with RVFV infection in legs and wings among FEF with infected bodies. TR is the proportion of FEF with RVFV positive saliva among FEF with disseminated infection Brustolin et al., 2017. TE is the percentage of FEF with infectious saliva among all the FEF (Jupille et al., 2016).

Alphavirus, flavivirus and phlebovirus detection

As previous studies revealed arboviral circulation in the study area (Alba et al., 2013), female mosquitoes, which were subjected to artificial blood meals and intrathoracic inoculations, were screened by reverse transcription nested polymerase chain reactions (RT-nPCR) to detect phlebovirus (family *Phenuiviridae*) (Sánchez-Seco et al., 2003), flavivirus (family *Flaviviridae*) (Sánchez-Seco et al., 2005) and alphavirus (family *Togaviridae*) (Sánchez-Seco et al., 2001) natural infections. Amplified flavivirus NS5 gene fragments were purified, sequenced and submitted to a basic local alignment search tool (BLAST) query for taxonomic assignation. To discard a virus insertion in the mosquito genome, DNA extracts from the samples that tested positive for flavivirus were treated with Ribonuclease A (RNase A) (Sigma-Aldrich, St. Louis, MO, USA) (Vázquez et al., 2012) prior flavivirus PCR amplification.

Statistics

In order to assess whether the natural infection of the Mosquito flavivirus influenced the vector competence for RVFV, the proportions of RVFV-infected mosquitoes in both Mosquito flavivirus-positive and Mosquito flavivirus-negative groups were compared using the Fisher's exact test (McDonald, 2009). Furthermore, we evaluated the differences in the mean RVFV Cq values of infected specimens depending on the presence/absence of the Mosquito flavivirus with a Wilcoxon test. All statistical analyses were carried out using R statistical software (http://cran.r-proje ct.org/).

RESULTS

Aedes vexans feeding and mortality rates

Four hundred and twenty-two and 148 *Ae. vexans* females emerged from field-collected larvae in 2016 and 2019, respectively. Low feeding rates (FR) were obtained after artificial blood meals [FR1 = 6.4% (27/422); FR2 = 12.6% (50/395); FR3 = 19.6% (29/148)].

In 2016, a mortality rate of 3.9% (3/77) was observed after blood-feeding; two and one deceased specimens exposed to RVFV and negative control groups, respectively. Meanwhile, in 2019 the mortality rates observed were 13.8% (4/29) and 21% (17/81) in females, which were orally exposed to RVFV and females subjected to intrathoracic inoculations, respectively.

Flavivirus detection in the field-collected *Aedes vexans* population

In 2016, flavivirus RT-nPCR showed a 58.4% (45/77) natural infection with a Mosquito flavivirus (71-nucleotide fragment; 99% similarity with OcFV137A_09, GenBank: JN257977.1). A similar prevalence of the Mosquito flavivirus (53.9%; 48/89) was observed for this mosquito population in 2019. Ribonuclease A (RNase A)-treated DNA extracts were negative for flavivirus by RT-nPCR discarding viral genome insertions. Alphavirus and phlebovirus screening excluded natural infection in the studied *Ae. vexans* population.

Vector competence of *Aedes vexans* for Rift Valley fever phlebovirus after oral exposure

Vector competence estimators evidenced that the RVFV infectious dose used in the present study (7.5 $\log_{10}TCID_{50}$/ml in infectious blood) allowed the virus to infect the body, disseminate through the haemolymph and be transmitted by field-captured *Ae. vexans* mosquitoes (**Tables 1, 2**). Only 17.7% (8/45) of the mosquitoes naturally infected with flavivirus resulted in infection with RVFV in contrast to 30% (6/20) of non-flavivirus-infected mosquitoes (**Table 1**).

However, given the small sample size, differences were not significant (P = 0.33). Additionally, no difference (P = 1) in the mean RVFV Cq values of infected specimens was observed between groups, with and without Mosquito flavivirus (**Figure 1**).

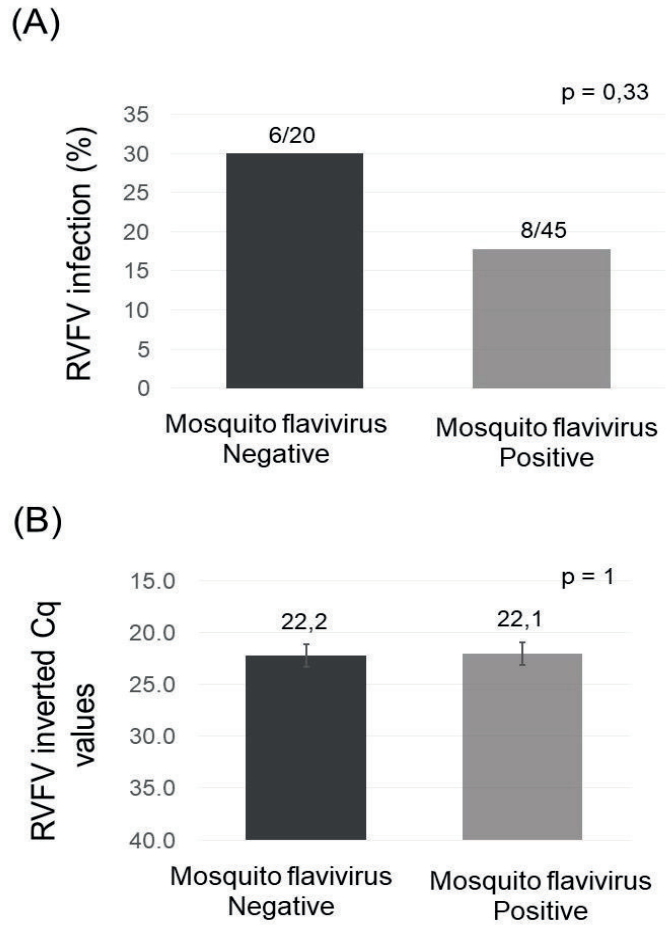

Figure 1. RVFV infection of *Aedes vexans* in relation to the presence or absence of a Mosquito flavivirus natural infection. **(A)** The proportion of RVFV infection is lower in mosquitoes naturally infected with a Mosquito flavivirus than in naturally non-infected mosquitoes. **(B)** RVFV mean Cq values in female bodies did not differ significantly in both groups, Mosquito flavivirus infected and non-infected. RVFV loads were not influenced by the Mosquito flavivirus infection.

Table 1. Vector competence of *Aedes vexans* for Rift Valley fever phlebovirus at 14 dpe. Infection, dissemination and transmission rates, and transmission efficiency of a natural *Ae. vexans* population from Catalonia, Spain orally exposed to Rift valley fever phlebovirus (RVFV 56/74)

	Feeding 1					**Feeding 2**					**Feeding 3**				
Mosquito flavivirus infection status	***n***	**IR**	**DIR**	**TR**	**TE**	***n***	**IR**	**DIR**	**TR**	**TE**	***n***	**IR**	**DIR**	**TR**	**TE**
Mosquito flavivirus negative (%)	4	0/4 (0)	0/0 (0)	0/0 (0)	0/4 (0)	11	5/11 (45)	1/5 (20)	1/1 (100)	1/11 (9.1)	5	1/5 (20)	1/1 (100)	1/1 (100)	1/5 (20)
Mosquito flavivirus positive (%)	12	1/12 (8.3)	1/1 (100)	0/1 (0)	0/12 (0)	23	3/23 (13)	1/3 (33.3)	1/1 (100)	1/23 (4.3)	10	4/10 (40)	2/4 (50)	2/2 (100)	2/10 (20)
Total (%)	16	1/16 (6.3)	1/1 (100)	0/1 (0)	0/16 (0)	34	8/34 (23.5)	2/8 (25)	2/2 (100)	2/34 (5.8)	15	5/15 (33.3)	3/5 (60)	3/3 (100)	3/15 (20)

Notes: IR, positive bodies/total fully engorged females; DIR, positive legs and wings/positive bodies; TR, positive saliva/ positive legs and wings; TE, positive saliva/total fully engorged females. *Abbreviations*: *n*, total fully engorged females; IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency

Table 2. Relevance of the midgut and salivary glands barriers in *Aedes vexans* after oral exposure to RVFV 56/74

	IR MIB	DIR MEB	TR SB	TE	Overall vector competence
Relative importance	14/65 (21.5%) +++	6/14 (42.9%) ++	5/6 (83.3%) null[a]	5/65 (7.7%)	Low

a Uncertain given the small sample size

Notes: Rating of relative importance of the barrier: null, virus crosses this barrier in >80% of females; +, minor, virus crosses this barrier in 60–80% of females; ++, moderate, virus crosses this barrier in 40–60% of females; +++, severe, virus crosses this barrier in 20–40% of females; ++++, very severe, virus crosses this barrier in < 20% of females [10]

Abbreviations: IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency; MIB, midgut infection barrier; MEB, midgut escape barrier; SB, salivary gland barrier

Out of six specimens with disseminated infection, five tested positive for RVFV in saliva (TR of 83.3%) by RTqPCR (Cq values: 22.38–33.94). The viability of RVFV viral particles of all these samples was confirmed by the cytopathic effect observed after incubation on Vero cell monolayers. Of the females which were able to transmit RVFV, three belonged to the Mosquito flavivirus naturally infected group; and two, to the non-infected group. For this *Ae. vexans* population, a transmission efficiency (TE) of 7.7% (5/65) was estimated.

Evaluation of salivary gland barriers of *Aedes vexans* for Rift Valley fever phlebovirus after intrathoracic inoculation

At day seven post-inoculation (7 dpi), RVFV dissemination and infection in all the specimens subjected to intrathoracic inoculations were confirmed (DIR = 100%, 45/45 and IR = 100%, 45/45). All the saliva samples that tested positive for RVFV by RT-qPCR (37/45; Cq = 23.89–33.34) also showed cytopathic effect after incubation on Vero cell monolayers. An 82.2% transmission rate was estimated, out of 45 inoculated specimens, 37 were able to transmit the virus.

DISCUSSION

To our knowledge, the present study reports for the first time a European field population of *Ae. vexans* as a competent vector for RVFV. In our study, oral exposure to the virulent strain RVFV 56/74 (7.5 $\log_{10}TCID_{50}$/ml in infectious blood) denoted severe and moderate importance of the *Ae. vexans* midgut infection and escape barriers, respectively; the virus was unable to cross these barriers in 78.5% and 51.1% in the overall FEF in each case. Meanwhile, the salivary gland barriers seem to be less important when a disseminated infection has already occurred. In the present study, transmission rates after oral exposure to the virus (83.3%) and after intrathoracic microinjections (82.2%) indicate that once RVFV is circulating through the haemocoel it is capable of successfully infecting the salivary glands and can transmit through the mosquito saliva.

Our overall results suggest that the studied population of *Ae. vexans* exhibits a low vector competence for RVFV (TE of 7.7%). Similarly, a German *Ae. vexans* laboratory colony was categorized as a low competent vector when orally exposed to infectious blood meals containing the virulent ZH548 strain and the avirulent Clone 13 strain (Moutailler et al., 2008). Previous studies have shown that *Ae. vexans* infection susceptibility and vector competence for RVFV is heterogeneous among geographically separated populations. In Senegal, for example, F1 specimens exposed to infectious blood meals containing three African strains (ArD141967, AnD133719 and SHM172805: at 4.5–9.5 × 106 PFU), exhibited moderate significance of the MIB, MEB and salivary gland barriers (IR: 30–85%; DR: 10.5–37%; and TR: 13–33.3%) (Ndiaye et al., 2016). These results were in accordance with several studies conducted at the USA where field captured specimens were subjected to oral exposure to viraemic animals inoculated with a variety of ZH501 strain doses (104.1–10.2PFU/ml) (Turell et al., 2008, 2013). In all cases, Senegalese and USA *Ae. vexans* populations showed a moderate RVFV vector competence. In contrast, studies that included populations from Canada (Iranpour et al., 2011),

California and Colorado (Turell et al., 2010), where field *Ae. vexans* populations were exposed to highly viraemic animals, revealed an inability to disseminate and transmit RVFV, respectively. Divergent results, besides the mosquito populations, could also be explained by differences in the viral strains or the infection methodologies used in each case.

The finding that the autochthonous population of *Ae. vexans* studied was naturally infected with a field-circulating Mosquito flavivirus, and it was maintained in the field through the years, was an interesting outcome of the experiment. The prevalence of this Mosquito flavivirus was consistent in both sampling years. Regarding RVFV co-infection with the circulating Mosquito flavivirus, our results show that the presence of the Mosquito flavivirus seemed to decrease the susceptibility to RVFV infection, although this effect was not statistically significant. Contrasting results were observed in our previous study (Talavera et al., 2018). The vector competence of a *Culex pipiens* colony, which was previously infected intrathoracically with Culex Flavivirus (CxFV), for the same RVFV strain (RVFV 56/74) was not affected by the infection with the CxFV. Diverse outcomes have been observed in several co-infection studies involving an insect-specific virus and a pathogenic one. For instance, in Colorado, *Cx. pipiens* naturally infected with CxFV showed a possible suppression in West Nile virus (WNV) early infection (Bolling et al., 2012). A similar co-infection, in *Culex quinquefasciatus* from Honduras, had the opposite effect, an enhancement of WNV transmission (Kent et al., 2010). *Aedes triseriatus* turned out to be resistant to Snowshoe hare virus infection in presence of LaCrosse virus, a closely related bunyavirus (Beaty et al., 1985). Further studies are required to clarify the potential role of the Mosquito flavivirus in the infection susceptibility and transmission of RVFV in the *Ae. vexans* population studied.

Finally, the experimental confirmation of a European biting nuisance species, such as *Ae. vexans*, as a RVFV vector highlights the necessity of regular and exhaustive arboviral vector surveillance and control strategies in

susceptible areas in the Mediterranean region, where *Ae. vexans* is distributed, to avoid a possible outbreak in the case of RVFV introduction.

CONCLUSIONS

The present study demonstrates for the first time that a European field-collected population of *Ae. vexans* may be involved in the transmission of RVFV in case of introduction to the continent. This knowledge contributes to the development of more accurate strategies for vector surveillance and control of RVF. The naturally circulating Mosquito flavivirus seems to modulate the susceptibility to RVFV infection in the assessed population of *Ae. vexans*. Further studies are needed to elucidate the potential of insect-specific viruses for the development of new biotools for the control of sanitary relevant arboviruses and their vectors.

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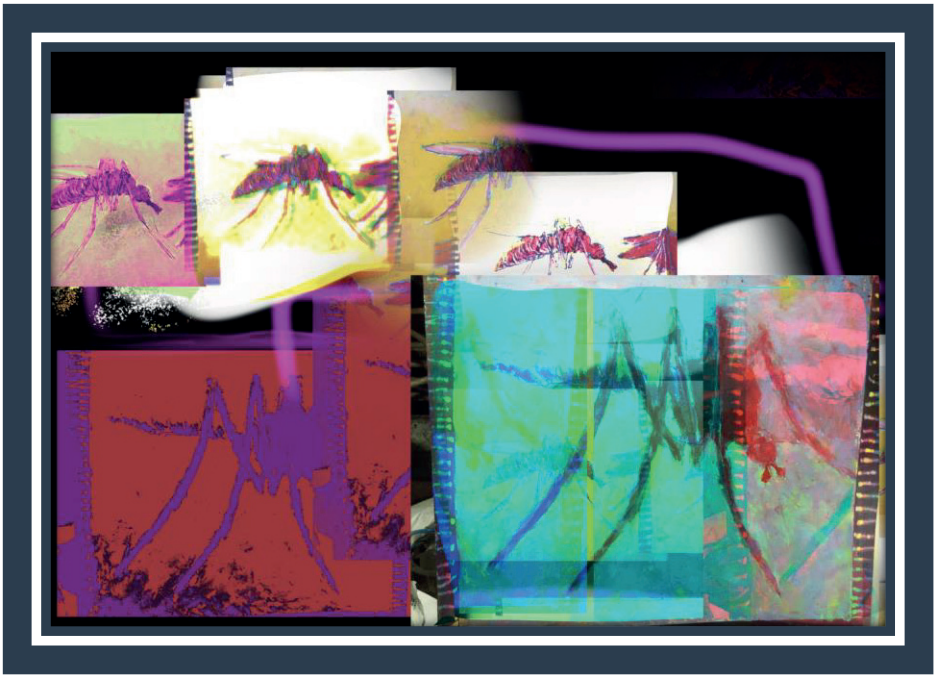

(Fragment original illustration: Abraham Birnberg, 2021)

CHAPTER 3

Metagenomics:
New Insights for Virus Detection

ASSAY 5

Viromics on Honey-Baited FTA Cards as a New Tool for the Detection of Circulating Viruses in Mosquitoes

Birnberg, L.; Temmam, S.; Aranda, C.; Correa-Fiz, F.; Talavera, S.; Bigot, T.; Eloit, M. and Busquets, N. *Viruses* (2020), 12, 274. doi: 10.3390/v12030274.

ABSTRACT

Worldwide, emerging and re-emerging infectious diseases (EIDs) are a major burden on public and animal health. Arthropod vectors, with mosquitoes being the main contributors of global disease, transmit more than 70% of the recognized EIDs.

To assess new alternatives for arthropod-borne viral diseases surveillance, and for the detection of new viruses, honey-baited Flinders Technology Associates (FTA) cards were used as sugar bait in mosquito traps during entomological surveys at the Llobregat River Delta (Catalonia, Spain). Next generation sequencing (NGS) metagenomics analysis was applied on honey-baited FTA cards, which had been exposed to field-captured mosquitoes to characterize their associated virome.

Arthropod- and plant-infecting viruses governed the virome profile on FTA cards. Twelve near-complete viral genomes were successfully obtained, suggesting good quality preservation of viral RNAs. Mosquito pools linked to the FTA cards were screened for the detection of mosquito-associated viruses by specific RT-PCRs to confirm the presence of these viruses. The circulation of viruses related to *Alphamesonivirus, Quaranjavirus* and unclassified *Bunyavirales* was detected in mosquitoes, and phylogenetic analyses revealed their similarities to viruses previously reported in other continents. To the best our knowledge, our findings constitute the first distribution record of these viruses in European mosquitoes and the first hint of insect-specific viruses in mosquitoes' saliva in field conditions, demonstrating the feasibility of this approach to monitor the transmissible fraction of the mosquitoes' virome.

In conclusion, this pilot viromics study on honey-baited FTA cards was shown to be a valid approach for the detection of viruses circulating in mosquitoes, thereby setting up an alternative tool for arbovirus surveillance and control programs.

Keywords: FTA cards; NGS; insect specific virus; saliva; *Alphamesonivirus*; *Quaranjavirus*; unclassified *Bunyavirales*.

BACKGROUND

Worldwide, two-thirds of all recognized emerging and re-emerging infectious diseases (EIDs) are of viral origin (Nii-Trebi, 2017), with arthropod-borne viruses (arboviruses) being the causative agents of more than 30% of them (Hollidge et al., 2010). Arboviruses circulate naturally between their vertebrate hosts and vectors. Nearly 135 arboviruses are known to infect humans, posing a significant threat to public health (Gubler, 2001). Globalization together with anthropic activities and climate change, has facilitated the dispersal of pathogenic agents (arboviruses included), their hosts and vectors, extending the risk to more and newer areas (Mayer, 2017; Franklinos et al., 2019). Since the increased incidence of dengue (Messina et al., 2014), Zika (Vest, 2016; Zinszer et al., 2017), chikungunya (Caglioti et al., 2013; Weaver and Forrester, 2015) and West Nile viruses (Gubler, 2007), there is a growing interest in understanding the viral diversity harbored by arthropod vectors, and a rising necessity to develop more effective surveillance and monitoring tools for circulating viruses.

Traditionally, for active surveillance and control purposes, samples from entomological surveys and/or from sentinel animals are subjected to laboratorial analyses to evidence arbovirus circulation. Despite these methodologies being considered the "gold standards", many issues must be considered. For instance, in entomological surveys, specialized personnel are required to capture and classify specimens, and a cold chain must be maintained to prevent virus degradation until molecular processing (Ritchie et al., 2013; Melanson et al., 2017). Due to the low prevalence of infected individuals between inter-epidemic periods, large numbers of mosquitoes have to be analyzed to detect a virus (Ritchie et al., 2013). When using sentinel animals, besides the necessary logistics, ethical considerations have to be taken into account, as the physical integrity of the animals, as well as that of the personnel, should be warranted (Johnson et al., 2015). Likewise, customary laboratorial techniques for virus detection present some limitations, for example in serological diagnosis, closely related viruses may produce cross-reactions (Johnson et al., 2015), while

PCR-based techniques target only those viral lineages that are already known, thereby underestimating the diversity of the sample while overlooking undescribed viruses that could potentially be pathogenic (Zheng et al., 2017).

Since gold-standard strategies are time-consuming, logistically complex and potentially hazardous, honey-baited Flinders Technology Associates (FTA) cards have been used as an alternative tool for arbovirus surveillance as they inactivate pathogens and preserve nucleic acids on contact, thereby simplifying the labor (Hall-Mendelin et al., 2010; Ritchie et al., 2013; Melanson et al., 2017). In previous field trials, honey-soaked FTA cards have been used in combination with molecular techniques to detect several arboviruses, such as Ross River virus (RRV), Barmah Forest virus (BFV) (Hall-Mendelin et al., 2010; Ritchie et al., 2013; Van der Hurk et al., 2014; Flies et al., 2015) and West Nile virus strain Kunjin (WNVKUN) (Ritchie et al., 2013; Van der Hurk et al., 2014) in Australia, and Usutu virus (USUV) in Switzerland (Wipf et al., 2019). Moreover, while virological surveillance in mosquitoes is based mainly upon virus detection in entire mosquitoes, indicating that they might be infected, the detection of viruses expectorated within the saliva during sugar feeding and deposited directly on the FTA cards may identify infectious mosquitoes (Flies et al., 2015).

To overcome the detection bias of molecular-based techniques, deep sequencing technologies have been proven as a valid approach to detect, characterize and discover unknown or uncultured viruses within biological or environmental samples (Delwart, 2007; Kristensen et al., 2010; Bibby, 2013; Greninger, 2018). Recently, by high throughput sequencing, diverse and widely distributed novel non-taxonomic groups of RNA viruses that naturally infect insects have been discovered in mosquitoes. Between 2007 and 2017, 187 novel mosquito-associated viruses have been reported and classified within 25 families (Atoni et al., 2019); some of them commonly grouped with human/animal arboviral pathogens or plant viruses. The capacity to detect

untargeted viruses enables metagenomics to act as a new and powerful approach to enhancing arbovirus surveillance programs (Batovska et al., 2019).

To the best of our knowledge, for the first time, next generation sequencing (NGS) on honey-impregnated FTA cards used as sugar bait during entomological surveys has been tested as a new approach for the detection of viruses circulating in mosquitoes. Viromics results on FTA cards were confirmed by the detection of mosquito-associated viruses in field-captured mosquitoes. Additionally, near-complete viral genomes were obtained. Herein, we show that insect-specific viruses (ISVs) can be detected in saliva from field-captured mosquitoes and report some ISVs previously identified in other continents, as first-distribution records in European mosquitoes.

MATERIALS AND METHODS

Study Area and Sampling Strategy

The present study was conducted at the Llobregat River Delta, North-Eastern Spain. In this Delta, densely populated areas coexist with natural habitats that serve as a strategic stopover on the route of migratory birds between Europe and Africa. For this reason, this area is considered to be of particular epidemiological interest and is targeted for arbovirus surveillance. In fact, sampling locations were chosen based on previous evidence of arbovirus circulation (Busquets et al., 2008), and in places where the *Servei de Control de Mosquits del Baix Llobregat* performs regular mosquito monitoring and control activities. Peri-urban and rural biotopes within this area were sampled to provide variability and increase the probability of virus detection.

Every fortnight, from May to November 2015, host-seeking female mosquitoes were captured using CO_2-baited EVS Mosquito Traps (Bioquip, Compton, CA, USA). Inside the collection bag of some traps, one honey-soaked Classic FTA™ card (Whatman™, GE Healthcare UK limited,

Buckinghamshire, UK) was placed as a sugar-bait for the captured specimens. Only the honey-impregnated area of the card was left exposed to allow specimens feed on it while in the trap (Van der Hurk et al., 2012). At each location, traps with and without honey-baited FTA cards were placed indiscriminately and kept operational from the early evening to the next morning (approximately 18 h). After sampling periods, FTA cards were removed, covered with Parafilm® (Bemis, Neenah, WI, USA) and coded according to location and sampling date. Only captured female mosquitoes were morphologically classified (Schaffner et al., 2001) and up to 30 individuals were pooled according to species, location and sampling date. A few non-culicid dipterans were also captured but not classified. The number of specimens with blue abdomens was recorded per species as evidence of feeding on the FTA cards. A cold chain was maintained through specimen transportation and handling to avoid RNA degradation (Van der Hurk et al., 2012). FTA cards and specimens were preserved at -80 °C until molecular analysis.

RNA Extraction from FTA cards for NGS Analysis

Pre-extraction, frozen FTA cards were thawed at 4 °C, homogenized with 500 µL of cold sterile PBS by vortex and squeezed with a sterile pestle to extract its content. Total RNA was obtained from individual FTA cards (13 peri-urban and 23 rural) using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was eluted in 50 µL of RNase-free water. A unique RNA sample per biotope was generated by pooling 15 µL of all the corresponding extracts of the given area.

Library Preparation, Sequencing and Bioinformatics Analysis

RNA samples were sequenced and analyzed as previously described with slight modifications (Moutailler et al., 2016). Briefly, to obtain complementary DNA (cDNA), RNA samples were retro-transcribed using random hexamers and the SuperScript IV reverse transcriptase (Invitrogen, Vilnius, Lithuania). Random amplification of cDNAs was performed using the multiple displacement

amplification (MDA) protocol with phi29 polymerase and random hexamers (Vayssier-Taussat et al., 2013). Libraries were sequenced at a depth of 60 to 80 million reads on an Illumina HiSeq2000 platform in a 150-base pairs (bp) single read format, outsourced to DNAvision Company (Charleroi, Belgium).

Raw reads were processed with an in-house bioinformatics pipeline as previously described (Temmam et al., 2019). Summarizing, it comprised quality check and trimming based on AlienTrimmer package (Criscuolo and Brisse, 2014) (Phred quality score cutoff = 80, min % of correctly called nt = 20) followed by read normalization using BBnorm program (https://jgi.doe.gov/data-and-tools/bbtools) (cut-off parameter of 100). *De novo* assemblies were performed using Megahit tool (Li et al., 2015) (minimum contig length = 100 nt). For further ORF prediction ((https://figshare.com/articles/translateReads_py/7588592), minimum aa length = 15), a Diamond-based similarity search (v0.9.22.123) against the protein Reference Viral database (RVDB-prot 16.0 (Bigot et al., 2019)) was conducted. Validation of viral taxonomic assignations was accomplished by a first Diamond-based search against the whole protein NCBI/nr database (1 November 2019 version) and a final search against the whole NCBI/nt nucleotide database (15 August 2019 version) to discard any putative non-viral intronic sequences that would, by chance, present a significant similarity with a viral protein. The pipeline used performs a protein blast for each viral contig and singleton, and then analyzes the taxonomic classification for all the co-best hits (meaning all the hits that have the same score). If all the hits were assigned to the same species, this species was reported as the closest hit. If the assembly had two or more different species or genera classifications, the last common ancestor was reported—genus or family, respectively. For low-level identities, taxonomic assignations were suggestive of putative new viral sequences. The quantification of abundance of each viral taxon was obtained by summing the length (in nucleotides) of all sequences being associated to this taxon, weighted by the *k*-mer coverage of each contig.

Primers Design and Virus Detection by Specific RT-PCRs

To confirm that viruses reported by metagenomics on FTA cards come solely from the captured specimens and not from the honey-bait, sequences assigned to mosquito-associated viruses were extracted. Among these, four viruses, with at least one assembly longer than 1000 nucleotides (nts) and with an identity higher than 90% were selected. Then, primers were designed from the extracted sequences of each chosen virus and conventional virus-specific reverse transcription polymerase chain reactions (RT-PCR) were set up. Viral RNA from mosquito pools and honey-baited FTA cards, which had not been exposed to mosquitoes, were then extracted using NucleoSpin® RNA Virus kit (Macherey–Nagel, Düren, Germany) following the manufacturer's instructions. Using the OneStep RT-PCR kit (QIAGEN GmbH, Hilden, Germany), all the above-mentioned samples were screened for the detection of Alphamesonivirus 1, *Bunyaviridae* environmental sample, Dezidougou virus and Wuhan mosquito virus 7, adjusting the annealing temperatures to each set of primers (**Table 1**). As positive amplification controls, Dezidougou virus isolate and Alphamesonivirus cDNA were used (kindly provided, respectively, by Scott Weaver from the World Reference Centre for Emerging Viruses and Arboviruses at University of Texas Medical Branch (WRCEVA–UTMB), and Patricia Gil and Serafín Gutiérrez from *Centre de Coopération Internationale en Recherche Agronomique pour le Développement* (CIRAD) at Montpellier). Meanwhile, for other viruses, since viral isolates were not available, extracted RNA from the FTAs that had been subjected to metagenomics were used as positive amplification controls. Amplification products were visualized in 2% agarose gels with ethidium bromide (0.1 µg/ml) staining.

Table 1. Virus-Specific Primers for RT-PCRs

Mosquito-associated viruses	Primer code	Primer nucleotide sequence (5' →3')	T_m (°C)	RT-PCR fragment size (bp)
Alphamesonivirus 1	ALPMF	GCGCCATTCTGCAGATCAAC	58	1033
	ALPMR	GTGCCAATAAACGCGTGATG		
Bunyaviridae environmental sample	BNYF	GAGTCCTTGTCCATCCCYGC	57	1059
	BNYR	GTGCAGGAAGAAGKAGCATGG		
Dezidougou virus	DZGF	GTCCTGTTAAGCTGCAACCC	56	400
	DZGR	CGTAACAACGATAAGTGGCG		
Wuhan mosquito virus 7	WHNF	GCGGAGAGAGGYAAAATGGATC	57	572
	WHNR	CATTCCCATCAGGAACCCTG		

Sequencing and Phylogenetic Analyses

Virus-specific RT-PCR products were purified using the QIAquick® Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and Sanger sequenced in both directions using the BigDye® Terminator v3.1 cycle Sequencing Kit (Life Technologies Corporation, Austin, TX, USA). At *the Servei de Genomica i Bioinformatica at the Universitat Autonoma de Barcelona* (SGB-UAB), amplicons were purified with the BigDye X Terminator kit (Applied Biosystems—,Waltham, MA, USA) and subjected to capillary electrophoresis in the Genetic Analyzer 3130xl (Applied Biosystems™, USA). Viral sequences were aligned using BioEdit Sequence Alignment Editor (Hall, 1999) and the identity of each virus was confirmed by comparing them to GenBank's reference database using the nucleotide Basic Local Alignment Search Tool (BLASTn) algorithm. At least one viral sequence per geographic region and a year that exhibited high similarities in the BLAST analysis to our subject sequences was used to infer the phylogenetic relationship of each studied virus. Viral sequences were then pairwise aligned using ClustalW algorithm in the Molecular Evolutionary Genetics Analysis program version X (MEGAX) (Kumar et al., 2018). In the same program, phylogenetic trees were constructed using the Maximum Likelihood (ML) method. Based on the Bayesian information criterion (BIC) score (Kumar et al., 2018; Nei and Kumar, 2000) the best models were applied. Tamura-Nei (TN93+G) with gamma distributions showed to be the best fit for Alphamesonivirus/CAT and Wuhan mosquito/CAT viruses, and Hasegawa-Kishino-Yano (HKY+G) (Hasegawa et al., 1985) with gamma distributions the best fit for *Culex* bunyavirus/CAT virus. In both cases, a 1000 replicate bootstrap was used.

Nucleotide Sequences Accession Numbers

The raw sequencing datasets for both batches of honey-baited FTA cards are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject ID: PRJNA604676 (www.ncbi.nlm.nih.gov/biosample/13978317

and www.ncbi.nlm.nih.gov/biosample/13978318). All the viral genomes for which the complete CDS were obtained were deposited in the GenBank archive under the accession numbers: MT096515-MT096531. Sequences corresponding to the viruses detected in mosquito pools from the Llobregat River Delta are available under the accession numbers: MT063093-MT063099.

RESULTS AND DISCUSSION

After sampling periods at the Llobregat River Delta, 1080 female mosquitoes were collected and classified into five species: *Aedes albopictus* (*n* = 20; 10 pools), *Coquillettidia richiardii* (*n* = 11; 5 pools), *Culex pipiens* (*n* = 755; 53 pools), *Aedes caspius* (*n* = 294; 24 pools) and *Aedes detritus* (*n* = 2; 1 pool) (**Table S1**). A total of 38 honey-baited FTA cards were recovered; 36 linked to mosquito captures and two from traps with no captures. Batches of 13 FTA cards from peri-urban and of 23 FTA cards from rural biotopes linked to mosquito captures constituted two independent samples for metagenomics analysis. Visual inspections depicted blue abdomens in 21% and 39% of the captured mosquitoes, respectively, for peri-urban and rural biotopes, confirming that they had fed on the FTA cards while in the trap. No evidence of blue dye was observed in *Ae. detritus* (**Table S1**).

Outputs on NGS on Honey-Baited FTA Cards

Next generation sequencing (NGS) on honey-baited FTA cards generated 61,362,209 and 80,631,320 of raw reads for rural and peri-urban biotopes, respectively. After filtering steps, 56,424,764 and 76,884,845 reads of 150 bases were assembled to produce 431,179 and 100,469 contigs respectively for rural and peri-urban datasets. Depurated reads also generated 3,128,224 and 846,017 singletons in each case.

Virome Composition on Honey-Baited FTA Cards during Entomological Surveys

Taxonomic assignations of the viral sequences obtained by high throughput sequencing on honey-baited FTA cards revealed that more than 95% corresponded to RNA viruses. *Picornavirales*, *Nidovirales* and *Tymovirales* were the most represented single-stranded positive sense RNA (ssRNA+) viral orders; and *Bunyavirales* the most abundant single-stranded negative sense RNA (ssRNA-) order. Double-stranded RNA (dsRNA) viral families *Partitiviridae* and *Totiviridae* were also dominant. DNA and unclassified viruses comprised the remaining 5% of the viral diversity herein reported (**Table S2**). In agreement with previous virome studies, most of the taxa derived from honey-baited FTA cards have been identified in various invertebrates (Shi et al., 2016) and associated to mosquitoes (Agboli et al., 2019). Additionally, mosquito-specific viruses detected in FTA cards (**Table 2**) have been described as part of the viral communities harbored by several mosquito species in different geographic regions (Frey et al., 2016; Sadeghi et al., 2018; Belda et al., 2019; de Oliveira et al., 2019; Öhlund et al., 2019; Pettersson et al., 2019; Sanborn et al., 2019; Shi et al., 2019).

Table 2. Mosquito-associated viruses identified in hone y-baited Flinders' Technology Associates (FTA) cards by next-generation sequencing (NGS) analysis. Taxonomic assignations with assembly lengths higher than 400 nt are shown. Abundance and contig length are expressed in nucleotides (nt). Viral identities are expressed in nucleotides and amino acids (aa).

	Closest hit	Gene/Product	Abundance	aa Identity (%)	Max. Contig length	% Coverage	nt Identity (%)	Accession No.
Rural	**Alphamesonivirus 1**	**Spike protein, hypothetical protein**	**3606196**	53-100	**1328**	**100**	**99.18**	**MF176279.1**
	***Bunyaviridae* environmental sample**	**RNA-dependent RNA polymerase**	**335292**	**49-99**	**4354**	**99**	**99.02**	**KP642114.1**
	Culex bunya-like virus	Hypothetical protein	289007	47-100	920	98	98.45	MH188002.1
	Culex iflavi-like virus 4	Polyprotein	1009175	71-100	1706	99	95.77	NC_040574.1
	Culex picorna-like virus 1	Polyprotein	806998	64-100	1238	100	96.37	MH703059.1
	Culex-associated Luteo-like virus	Hypothetical protein, RNA-dependent RNA polymerase	3285	67-100	566	99	95.04	MK440647.1
	Dezidougou virus	Hypothetical protein 1	9366	87-100	638	100	94.34	KY968698.1
	Hubei picorna-like virus 61	Hypothetical protein	53578	84-100	916	99	95.63	KX883915.1
	Wenzhou soberno-like virus 4	Hypothetical proteins 1 and 2	668852	94-98	2284	100	96.67	KX882831.1
	Wuhan mosquito virus 5	PB1	5460	50	580	13	75.95	KX898491.1

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Peri-urban	*Aedes pseudoscutellaris* reovirus	VP1	5244	69-100	667	99	78.08	DQ087276.1
	Alphamesonivirus 1	ORF1a, pp1a polyprotein	22590	60-100	932	100	98.18	MH520106.1
	Culex Hubei-like virus	Hypothetical protein	5142	85-100	510	91	90.34	MH188025.1
	Culex iflavi-like virus 4	Polyprotein	168154	97-100	2170	100	96.04	NC_040574.1
	Culex luteo-like virus	RdRp	16686	42-67	1279	65	67.49	MF176386.1
	Culex picorna-like virus 1	Polyprotein	102979	77-100	1290	100	98.29	MH703059.1
	Culex pipiens associated Tunisia virus	Replicase	11319	96-100	1446	98	89.11	NC_040723.1
	Culicine-associated Z virus	VP1, RdRp	14584	77-97	765	96	83.33	KF298283.1
	Daeseongdong virus 1	ORF1, putative RNA-dependent RNA polymerase	614537	75-95	5831	95	82.27	KU095841.1
	Dezidougou virus	**Hypothetical protein 1**	**1424472**	**85-100**	**1882**	**100**	**95.42**	**KY968698.1**
	Karumba virus	Similar NS5 protein	96687	49	3160	28	76.31	JF707857.1
	Hubei picorna-like virus 61	Hypothetical protein	5815018	70-100	1252	100	96.01	KX883915.1
	Negevirus nona 1	Hypothetical protein	190830	49-95	2765	99	87.11	AB972669.1
	Wuhan mosquito virus 6	Nucleoprotein	9480	72-100	468	100	97.01	MF176381.1
	Wuhan mosquito virus 7	**PB1**	**43351**	**53-100**	**1846**	**100**	**92.15**	**KM817626.1**

Bold type corresponds to the selected viruses for primers design.

In the present study, taxonomic profiling revealed the prevalence of invertebrate-associated viruses (**Figure 1A**) with *Dicistroviridae, Iflaviridae* and *Mesoniviridae* being the most abundant families (**Figure 1B**). Sequences herein designated as *Dicistroviridae* and *Iflaviridae* (order *Picornavirales*) were mostly related to hymenopterans, in particular to the honeybee *Apis mellifera*. Since we could not sequence the honey used to impregnate the FTA cards as sugar bait, we cannot discard the possibility that these sequences might have come from it. However, recent virome studies have described these two families as the most abundant in culicid mosquitoes from the Yunnan province in China, and Zambezi province in Mozambique (Atoni et al., 2018; Cholleti et al., 2018). The additional description of honeybee-infecting virus *Rhopalosiphum padi* virus (*Dicistroviridae*, genus *Cripavirus*) in mosquito species from Hubei, China (Shi et al., 2015) and in *Culex* mosquitoes from California (Sadeghi et al., 2018), together with the assembly of sequences linked to chronic bee paralysis virus (CBPV) (unclassified ssRNA+ virus) and *Apis mellifera* filamentous virus (dsDNA *Hytrosaviridae* family) from French *Anopheles maculipennis* (Cook et al., 2013) and from *Culex* mosquitoes from California (Sadeghi et al., 2018) respectively, suggested that these viral families could be associated to mosquitoes as well. In addition, due to the low genetic identity of these viruses with their closest honeybee counterpart, the scarcity of mosquito-based sequences available in public databases, and the continuous discovery of new picorna-like viruses in insects (Sadeghi et al., 2018; Öhlund et al., 2019; Cholleti et al., 2018; Xia et al., 2018; Habayeb et al., 2019), might suggest that we are dealing with novel mosquito picorna-like viruses. Based on the abovementioned findings, captured mosquitoes that fed on the FTA cards could have been the source of the identified viruses.

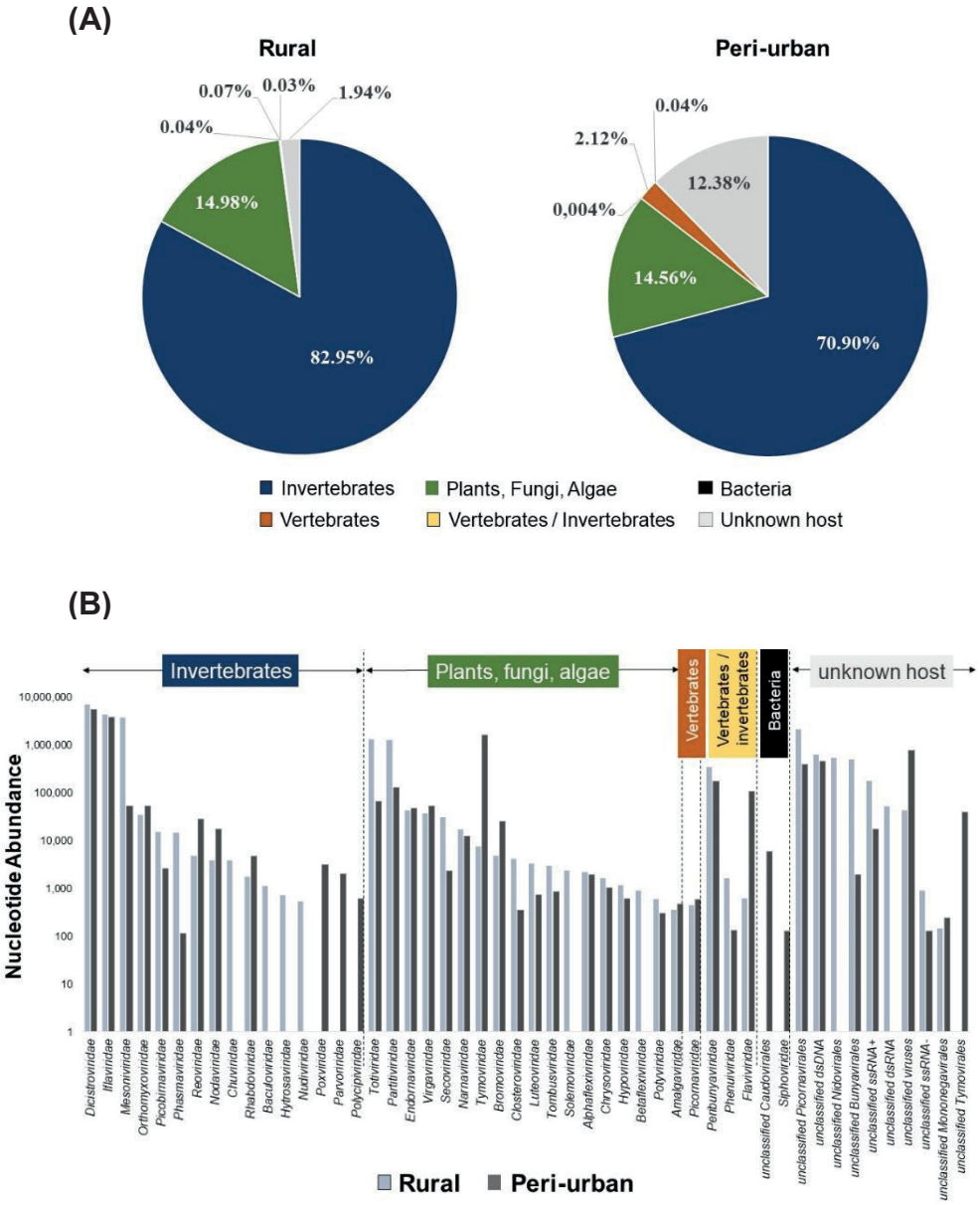

Figure 1. Overview of viral composition of honey-baited FTA cards. (**A**) Shows the proportion of viral reads classified by host type. Proportions of bacteria and vertebrate/invertebrate are too small to be seen in the figure. (**B**) Abundance in nucleotides of each viral family estimated by summing sequence length in nucleotides weighted by the k-mer coverage of each contig.

Besides invertebrate-related viruses, it was not surprising to find viral families usually detected in plants, fungi and algae (e.g., *Tymoviridae*, *Totiviridae*, *Partitiviridae*, *Endornaviridae* or *Virgaviridae*) as part of the viral diversity associated to honey-baited FTA cards (**Figure 1B**). Since, in *Culex* mosquitoes, sequences related to *Totiviridae*-like viruses have been found in Guadeloupe (Shi et al., 2019), Australia (Batovska et al., 2019), China (Atoni et al., 2018) and California (Sadeghi et al., 2018); *Partitiviridae*-like viruses have been detected in Sweden (Öhlund et al., 2019; Petterson et al., 2019), Australia (Batovska et al., 2019), Kenya (Atoni et al., 2018) and California (Sadeghi et al., 2018); *Endornaviridae*-like viruses in Australia (Batovska et al., 2019) and *Tymoviridae*-like viruses have been identified in Guadeloupe (Shi et al., 2019), Kenya (Atoni et al., 2018), California (Sadeghi et al., 2018), China (Xia et al., 2018), and Sweden (Petterson et al., 2019). Moreover, a *Culex Tymoviridae*-like virus (CuTLV) that was isolated from a *Culex* spp. pool from Xinjiang (China) was also shown to produce a cytopathic effect on *Aedes albopictus* C6/36 cell line (Wang et al., 2012), suggesting a potential plant/mosquito host-shift even when there is no record of mosquitoes as vectors of plant viruses (Shi et al., 2019). Nonetheless, there is also the chance that: i) mosquitoes could have acquired these viruses while sap or nectar feeding prior to capture and deposited them on the FTA card along with saliva expectorations as mouthparts contaminants (Atoni et al., 2018; Forrester et al., 2014) while trapped; or ii) they could have been present in the honey used as bait.

To a lesser extent, the virome profile of FTA cards depicted sequences assigned to three dual-host (mosquito/vertebrate) virus families: *Flaviviridae*, *Phenuiviridae* and *Peribunyaviridae*. *Flaviviridae*-associated sequences were distantly related to two mosquito-specific viruses, Karumba virus (49% amino acid (aa) identity) and Calbertado virus (47–86% aa identities) (Table S2). Reads related to *Phenuiviridae* were assigned to a distant Phasi Charoen-like phasivirus with aa identities ranging from 58% to 77% (Table S2). Meanwhile, most of the *Peribunyaviridae*-associated sequences presented high homologies

with Ganda bee virus (35–95% aa identity) (**Table 2**). Finally, no arboviruses were detected throughout the sampling period by NGS on honey-baited FTA cards. Despite six sequences matched with WNV (59–92% aa identity), these assignations were not taken into consideration due to the length (150 nt), nucleotide identity (<80%) and coverage (<80%) of the sequences.

Viral Genomes Obtained from Honey-Baited FTA Cards

It is noteworthy that de novo assemblies of viral reads from both honey-baited FTA cards batches produced 12 near-complete viral genomes (>98% nucleotide coverage and >93% nucleotide identity) for which the 50 and 30 termini are incomplete since RACE-PCRs were not performed. Viral genomes within the orders *Nidovirales* (Alphamesonivirus 1: Ngewotan virus) and *Picornavirales* (e.g., Deformed wing virus and *Culex* Iflavi-like virus 4), and within unclassified RNA viruses (e.g., Hubei picorna-like virus 61 and Wenzhou soberno-like virus 4) were generated (**Table 3**). Obtaining near-complete genomes of viruses associated to mosquitoes, highlights the usefulness of FTA cards in preserving viral RNA. However, we cannot exclude that most of the honeybee-related virus genomes might come from the bait.

Table 3. Near-complete viral genomes obtained by NGS on honey-baited FTA cards. Viral assignations with a genome coverage higher than 98% and identities higher than 95% are shown.

Sample	Order	Family	Closest virus	No Reads	Mean coverage per nt	Coverage (%)	% Identity (nt)	Accession No
Rural	*Picornavirales*	*Dicistroviridae*	Kashmir bee virus	28080	401,29 X	100	96,74	AY275710.1
			Black queen cell virus isolate BQCV_MS	3112	49,85 X	100	93,78	MH267694.1
		Iflaviridae	Deformed wing virus isolate Hamilton	3921	51,47 X	100	99,77	MF623172.1
			Culex iflavi-like 4 virus strain CIVL/Kern	17787	250,75 X	100	95,78	NC_040574.1
	Nidovirales	*Mesoniviridae*	Ngewotan virus strain mos172×93828	9326	63,03 X	100	98,88	MF176279.1
		Unclassified RNA viruses	Wenzhou soberno-like virus 4 strain mosZJ35391	12059	562,28 X	99	96,79	KX882831.1
Peri-urban	*Picornavirales*	*Dicistroviridae*	Aphid lethal paralysis virus isolate ALPV-CE	572	8,42 X	99	94,75	JX480861.1
		Iflaviridae	Deformed wing virus isolate Hamilton	3670	47,57 X	100	99,75	MF623172.1
			Culex iflavi-like 4 virus strain CIVL/Kern	1435	20,74 X	100	95,72	NC_040574.1
		Unclassified RNA viruses	Hubei picorna-like virus 61 strain mosHB235903	147377	2384,82 X	100	95,84	KX883915.1
			Hubei noda-like virus 11 strain arthropodmix22482	210275	6 964,36 X	100	97,58	KX883010.1
			Dezidougou virus strain DEZI/*Aedes africanus*/SEN/DAK-AR-41524/1984	4939	74,39 X	98	95,32	KY968698.1

Virus Detection by Specific RT-PCRs on Honey-Baited FTA Cards Unexposed to Mosquitoes

To confirm virome results obtained through metagenomics analysis on honey-baited FTA cards, among all the mosquito-associated viruses (**Table 2**), Alphamesonivirus 1 (3.606.196 abundance in nucleotides), Dezidougou virus (1.424.472 abundance in nts), *Bunyaviridae* environmental sample (335.292 abundance in nts) and Wuhan mosquito virus 7 (43.351 abundance in nts) were selected to design specific primers and set up virus-specific RT-PCRs. All these selected viruses showed to have at least one contig with a matching sequence longer than 1000 nt and similarity above 90%. For identification matters, through the manuscript, these viruses would respectively be referred to as Alphamesonivirus/CAT virus, *Culex* bunyavirus/CAT virus, Dezidougou/CAT virus and Wuhan mosquito/CAT virus. Suffix "CAT" stands for the geographic region of detection, i.e., Catalonia.

Those honey-baited FTA cards, which were not exposed to mosquitoes recovered from entomological surveys, were then screened individually by virus-specific RT-PCRs to verify the source of the viruses detected by viromics. Screenings of both cards tested negative for *Culex* bunyavirus/CAT virus and Alphamesonivirus/CAT virus, and positive for Dezidougou/CAT virus and Wuhan mosquito/CAT virus. These detections could be explained by (i) the presence of non-culicid dipterans in the traps; they could have deposited these viruses while sugar feeding from the FTA cards, and/or (ii) the source of these viruses came from the honey impregnated on the cards.

Virus Detection by Specific RT-PCRs on Field-Captured Mosquito Pools

Virus-specific screenings on mosquito pools confirmed virus circulation as depicted by NGS on FTA cards (**Figure 2**). Throughout sampling periods, *Culex* bunyavirus/CAT virus (unclassified *Bunyavirales*) was the most common and was recurrently detected in both biotopes (**Figure 2**). Out of 53 *Cx. pipiens* pools, 50 were found to be infected (including 14 pools unexposed

to FTA cards), showing a high occurrence of this viral strain in *Cx. pipiens* mosquitoes from the Llobregat River Delta. BLASTn analysis of the amplified fragment of a RT-PCR positive pool showed a nucleotide similarity of 97.58% to *Bunyaviridae* environmental sample's RNA-dependent RNA polymerase gene (RdRp). Phylogenetically, our strain clustered with *Bunyaviridae* environmental sample (2013) and *Culex* Bunyavirus 2 (2016), which have previously been detected in *Culex* spp. mosquitoes from the United States of America (USA) (**Figure 3A**). The discovery of *Culex* bunyavirus/CAT virus in Catalonian *Cx. pipiens* widens the range of known distribution for this mosquito-specific bunyaviruses from the USA in California (Sadeghi et al., 2018; Chandler et al., 2015) and Maryland (Frey et al., 2016), to Spain. Our findings might also suggest that these bunyaviruses could be genus-specific, as they have been detected only in *Culex* spp. mosquitoes.

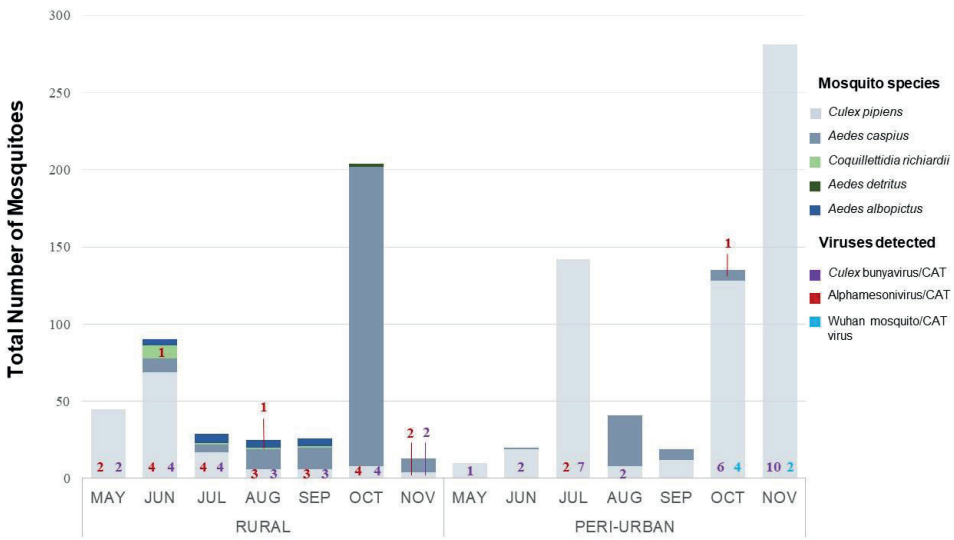

Figure 2. Mosquito species dynamics and virus occurrence in rural and peri-urban biotopes from the Llobregat River Delta. Cumulative bars represent the total number of female mosquitoes captured per month per sampling site. Numbers in color correspond to the total number of mosquito pools that tested positive for a given virus on a particular month and sampling site.

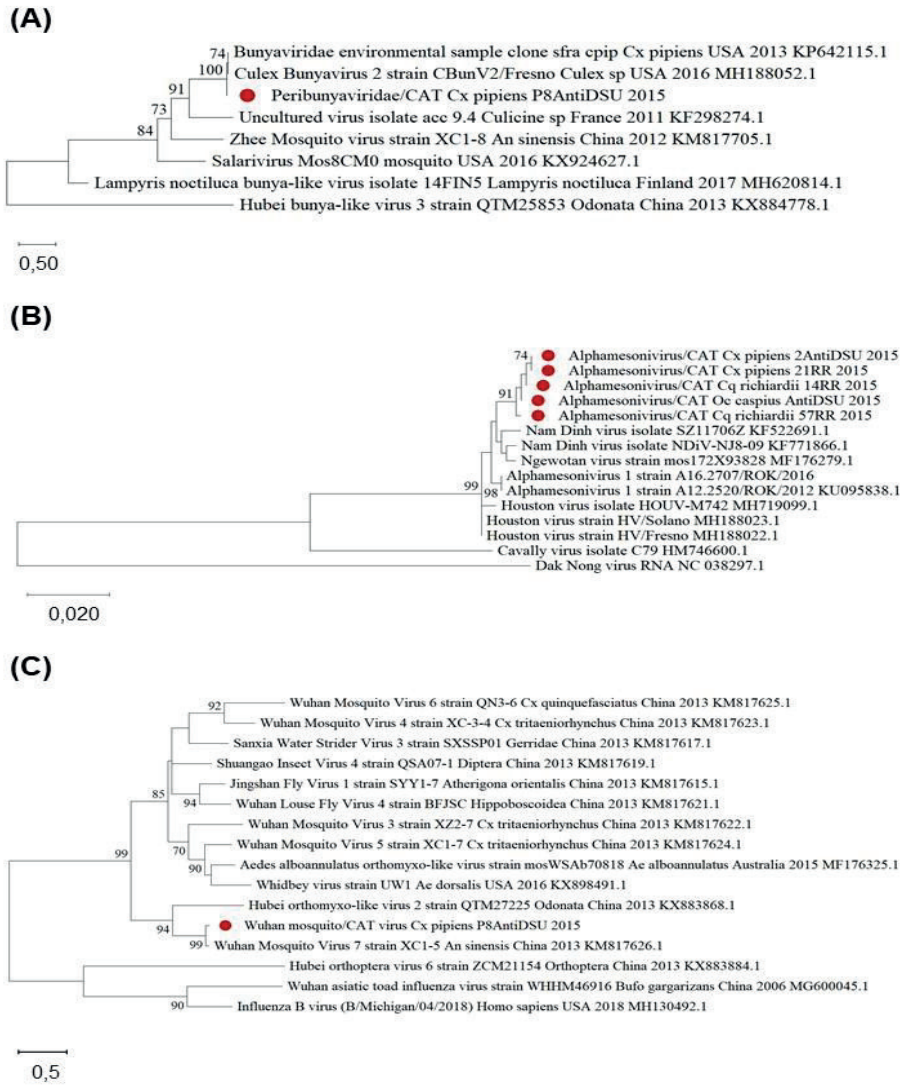

Figure 3. Phylogenetic trees of viruses detected by virus-specific RT-PCR in Catalonian mosquitoes. Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. Codon positions included were 1st+2nd+3rd+Noncoding. A discrete Gamma distribution was used to model evolutionary rate differences among sites. (**A**) *Culex* bunyavirus/CAT virus, evolutionary history inferred by using the Maximum

Likelihood (ML) method and Hasegawa-Kishino-Yano model (HKY+G). The tree with the highest log likelihood (-6117.36) is shown (five categories (+G, parameter = 1.2252)). There were 946 positions in the final dataset. (**B**) Alphamesonivirus/CAT evolutionary history inferred by using the ML method and Tamura-Nei (TN93+G) model. The tree with the highest log likelihood (-2006.57) is shown (five categories (+G, parameter = 0.3417)). There were a total of 839 positions in the final dataset. (**C)** Wuhan mosquito/CAT virus evolutionary history was inferred by using the ML method and Tamura-Nei model (TN93+G). The tree with the highest log likelihood (-8996.79) is shown (five categories (+G, parameter = 0.7704). There were a total of 729 positions in the final dataset.

Alphamesonivirus/CAT virus, the second most commonly detected virus (Figure 2), was identified in 24 *Cx. pipiens* pools, two *Cq. richiardii* and one *Ae. caspius* pools. *Alphamesonivirus* is the only recognized genus within the mosquito-restricted family *Mesoniviridae* (order *Nidovirales*) (Lauber et al. 2012). Strains herein reported, shared >98% nucleotide identity to Houston virus and Nam Dinh virus strains' open reading frame 2 (ORF2) and were closely related to several alphamesonivirus strains that have been detected between 2008 and 2016 in *Culex* spp. mosquitoes. Houston virus (HOUV) and Nam Dinh virus (NDiV) in *Culex quinquefasciatus* from Mexico and China; Ngewotan virus in *Culex australicus* from Australia; NDiV, Alphamesonivirus-1 and HOUV in *Culex* spp. from China, South Korea, and the USA. It is worth mentioning that the viral strains detected in the present study demonstrated a closer relationship to each other than to the strains found in other geographic regions. Moreover, Alphamesonivirus/CAT strains found in *Cx. pipiens*, both rural and peri-urban biotopes, appeared to be more closely related to each other than to those found in other mosquito species from the same geographic area (Figure 3B), thereby suggesting co-evolution events within their host species. These findings, together with the detection of an alphamesonivirus in *Cx. pipiens* from Camargue, France (Gil et al., 2017), confirm the wide geographical distribution and host range described for the family *Mesoniviridae* (Vasilakis et al., 2014). Recently, viruses belonging to this family have been continually detected by virome metagenomics approaches in several mosquito species

(Hang et al., 2016; Atoni et al., 2018; Sadeghi et al., 2018; Xia et al., 2018; Sanborn et al., 2019), therefore providing more support for this asseveration.

Finally, Wuhan mosquito/CAT virus was positively detected in six of 53 *Cx. pipiens* pools (**Figure 2**). Among these, five were captured in traps without honey-baited FTA cards and only one was exposed to a FTA card. Wuhan mosquito/CAT virus exhibited a high phylogenetic relationship (92.15% of nucleotide similarity) with Wuhan mosquito virus 7 strain's PB1 gene detected in *Anopheles sinensis* from China in 2013 (**Figure 3C**). Wuhan mosquito virus 7 belongs to *Quaranjavirus* genus (family *Orthomyxoviridae*, order *Articulavirales*), which has been identified in a pool of *Anopheles sinensis* and *Culex quinquefasciatus* mosquitoes originating from Hubei, China (Li et al., 2015). Finally, throughout screenings, neither *Ae. albopictus* nor *Ae. detritus* were found to be infected by any of those viruses targeted. Detecting *Culex* bunyavirus/CAT and Wuhan mosquito/CAT viruses in *Cx. pipiens* pools, which were not exposed to honey-baited FTA cards, evidenced that these viruses were indeed infecting the mosquitoes and were not acquired while sugar feeding on the FTA cards.

The discovery of *Culex* bunyavirus/CAT, Alphamesonivirus/CAT and Wuhan mosquito/CAT viruses in culicid mosquitoes found in Catalonia, contributes to the knowledge of both the host range and their geographical distribution.

Overall Remarks of the Approach and Future Perspectives

The current study is a pioneer in applying viromics on honey-baited FTA cards during entomological surveys as a tool for the detection of circulating viruses in mosquitoes and the identification of virus in mosquitoes' saliva. Through this approach, 19 ssRNA (+), six ssRNA (-), eight dsRNA, one ssDNA, five dsDNA viruses and several unclassified viruses were identified; and 12 near-complete viral genomes were obtained from FTA cards, among which seven were linked to mosquito species of sanitary relevance. Acquiring near-complete

virus genomes is a clear advantage of metagenomics over classical surveillance based on PCR detection, since insights into the origin, evolution, and diversity of circulating viruses could be gained (Batovska et al., 2019). Further detection of *Culex* bunyavirus/CAT virus, Alphamesonivirus/CAT virus and Wuhan mosquito/CAT virus in mosquito pools confirmed the presence of these viruses in Europe, where previously their circulation had not been revealed. These findings highlight the value of honey-baited FTA cards combined with viromics in identifying a wide spectrum of viruses that may be associated to sylvan mosquitoes in susceptible areas for arbovirus transmission, without requiring previous knowledge of viral diversity. In future arbovirus surveillance, NGS on honey-baited FTA cards could be used as a guide for prevention and control strategies. In the case of arboviruses detection, entomological surveillance could be exhaustively carried out focusing on specimen classification and molecular analysis where the virus of interest has been previously detected in the FTA cards.

It is worth mentioning that, in spite of the advantages provided by NGS on honey-baited FTA cards, there are some drawbacks that need to be mentioned. Firstly, since FTA cards inactivate the viruses, and NGS provides only genetic information through this approach, no viable virus could be isolated for further characterization. Secondly, virus-bearing mosquito species could not be identified without complementary morphological and molecular analyses. Other possible constraints of this approach could be related to the feeding rate on FTA cards, the quantity of saliva expectorated by mosquitoes, and the number of viral copies liberated within the saliva while sugar feeding. The assumption of blue abdomens in mosquitoes, as the only proof of virus expectoration on FTA cards, might possibly overlook virus release while probing. This fact was evidenced with the detection of chikungunya virus (CHIKV) RNA in FTA cards exposed to experimentally infected *Aedes aegypti* despite the fact that there not been any record of blue dye in their abdomens (Hall-Mendelin et al., 2010). Based on these findings, viruses identified by NGS

in FTAs could also have been deposited by mosquitoes in which blue abdomen were not present.

Furthermore, to improve the sensitivity and efficiency of our approach, honey-baited FTA cards could be placed inside Box gravid traps, as a recent study conducted in Switzerland demonstrated these to be the most effective traps for capturing females of different species when searching for an ovipositional site. In addition, these traps also exhibited the highest feeding success on honey-baited FTA cards (Wipf et al., 2019).

The detection of ISVs through metagenomics on honey-baited FTA cards provides evidence that these viruses could be transmitted within mosquitoes' expectorations, thereby contradicting previous beliefs that they could not be expelled with saliva (Wipf et al., 2019). Our findings are supported by the tissue tropism evidenced for *Culex* flavivirus (family *Flaviviridae*) and Phasi Charoen-like virus (PCLV) (genus *Phasivirus*, family *Phenuiviridae*), as they were also detected in salivary glands of *Cx. pipiens* from Iowa (Saiyasombat et al., 2011) and in *Ae. aegypti* from South China (Zhang et al., 2018), respectively, and, most importantly, by the detection of *Aedes* flavivirus RNA in saliva from colonized *Ae. albopictus* (Bolling et al., 2015). Sequences distantly related to PCLV were also detected in our FTA cards. To date, ISVs transmission seemed to be primarily vertical from the adult female to its progeny and venereal from males to females (Bolling et al., 2011; Saiyasombat et al., 2011). However, horizontal transmission has been hypothesized on breeding sites by direct contact, through feeding in larvae and adults, and/or by copula (Agboli et al., 2019). Further studies are required to assess the transmission dynamics of the ISVs herein identified.

Furthermore, ISVs are a significant part of the mosquito's virome. Due to their phylogenetic relationships, great abundance and high diversity, it is presumed that arboviruses might have been originated from arthropod-infecting viruses (Bolling et al., 2015; Dudas and Obbard, 2015; Öhlund et al., 2019). In

addition, these viral symbionts are thought to alter the mosquito's innate immune response, therefore modulating the vector competence for certain arboviruses, and so giving rise to new potential biotools for arbovirus control and prevention (Öhlund et al., 2019). For instance, *Culex* flavivirus naturally infecting *Cx. pipiens* from Colorado possibly suppressed the early infection with West Nile virus (WNV) (Bolling et al., 2012). In Thailand, Zika virus (ZIKV) and dengue virus 1 (DENV-1) titers in head tissues of *Aedes aegypti* were reduced by intrathoracic inoculation of newly isolated cell fusing agent virus (CFAV) (Baidaliuk et al., 2019). Likewise, a mosquito flavivirus of natural circulation in *Aedes vexans* from Catalonia seemed to decrease the susceptibility of infection to Rift Valley fever phlebovirus (RVFV) following experimental oral exposure (Birnberg et al., 2019).

As evidenced, and in spite of the continual discovery of novel mosquito-associated viruses, viral diversity harbored by vector species is still underestimated and little is known about their host range, distribution, ecology and evolution (Bolling et al., 2015; Vasilakis and Tesh, 2015). Further studies are required to isolate and fully characterize the genome of Alphamesonivirus/CAT, *Culex* bunyavirus/CAT and Wuhan mosquito/CAT viruses so as to assess their potential as vertebrate pathogens. Finding these ISVs in FTA cards, and therefore in mosquitoes' saliva, rises concerns of the potential of these viruses to evolve from being insect-specific to dual-host viruses, acquiring the ability to infect vertebrate cells and become new emerging pathogens. Future surveillance strategies for emerging diseases could include NGS on honey-baited FTA cards to detect previously undiscovered and potentially transmissible viruses so as to prevent new arbovirus outbreaks.

CONCLUSIONS

The detection of viruses related to Alphamesonivirus, Quaranjavirus (Wuhan mosquito virus), and unclassified *Bunyavirales* in European field-captured mosquitoes using virus-specific primers derived from metagenomics results, demonstrated that viromics on honey-baited FTA cards is a valid approach for virological surveillance in mosquitoes. To the best of our knowledge, this is the first evidence of circulating ISVs in mosquitoes' saliva under field conditions. Our study also constitutes the first distribution record of these viruses in the European continent, thereby demonstrating that they are widely distributed despite there being an information gap due to the majority of studies being focused primarily on arbovirus detection. Further studies are needed to better understand the evolutionary history of insect-specific viruses and their potential role in arbovirus transmission.

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Suppplementary Material

https://www.mdpi.com/1999-4915/12/3/274/s1,

Table S1: Species composition and feeding rates on honey-baited FTA cards in peri-urban and rural biotopes from the Llobregat River Delta

Table S2: Viral taxonomic assignations of assembled sequences associated to FTA cards linked to mosquito species during entomological surveys in Catalonia

GENERAL DISCUSSION

In a changing world in which mosquito-borne diseases (MBD) are still one of the major causes of morbidity and mortality in tropical and sub-tropical regions, globalization, anthropic activities, and climate change have increased the likelihood for their resurgence and/or introduction into non-endemic countries. Over the past decade, Europe has dealt with the continuous threat of the establishment and spread of (re)emerging MBD such as malaria and arboviral diseases, among which, RVF is a serious concern. Consequently, there is a growing interest to improve preparedness and response capacities to face with the future epidemiological setting.

For the occurrence of local MBD transmission certain factors must coincide, i) favorable environmental conditions for vector and pathogen development; ii) a vertebrate/human population that may act as a reservoir; iii) the presence of competent vector species to amplify and transmit the pathogen; and iv) the pathogen agent. Since in the European continent, a suitable environment and susceptible populations can be found, it is crucial to determine whether or not local mosquito populations (native and invasive) are able to acquire, sustain and transmit medically/veterinary relevant pathogens in case of introduction. In this regard, VC assays provide valuable information to assess the risk of outbreak or pathogen emergence.

Conventionally, VC assays are conducted under controlled laboratory conditions using field-caught and/or laboratory specimens, but sometimes contrasting results are obtained depending on mosquito population-pathogen-environmental conditions combination. For instance, populations of *Ae. vexans* from North America (Turell et al., 2010) and Central Europe (Moutallier et al., 2008), showed different abilities to transmit RVFV when compared to a Spanish population (Chapter 2, assay 4). In this way, VC for arbovirus transmission vary between species, different populations of the same species

(natural and/or laboratory), and between viral strains as demonstrated by a recent study that assessed the global VC potential of Zika vectors (Obadia et al.,2022), and by a meta-analysis that quantified the competence of the five major potential vectors of RVFV in the Mediterranean (Drouin et al., 2022). This fact has led to an increased interest for a better understanding of the extrinsic (e.g., climate conditions and virus strains) and intrinsic (e.g., genetics and microbiome) factors behind mosquito's infection susceptibility; and to interpret VC outcomes more accurately and improve the risk assessment for RVFV establishment and transmission in a specific region. In order to do this, the first step should be the proper identification/characterization of mosquito populations (sylvan or laboratory) since some species are highly polymorphic and sibling species (*Anopheles*) and/or forms (*Culex*) are morphologically no differentiable, but, their behavior, host preference and vector competence could be different (Farajollahi et al. 2011; Bennett et al., 2002). In the present thesis (Chapter 2, assay 3), *Culex pipiens molestus* and hybrid forms showed differences in terms of VC (although no statistically significant due to the sample size), with *Cx. pipiens molestus* being a competent vector for RVFV transmission, while *Cx. pipiens* hybrid was not. Factors that may confer the importance of the MEB in *Cx. pipiens* hybrid form should be thoroughly analyzed. As a second step, owing to the direct relationship between the fitness of vector mosquitoes and the microorganisms they harbor (Minard, Mavingui, and Valiente, (2013), a comprehensive profiling of the microbiome of local populations would increase the knowledge of the structure (composition and diversity) of their microbial communities and set baselines for further functional studies that address a better understanding of the potential role of these microorganisms in biological traits and vector competence of their hosts. In this regard, molecular techniques and high-throughput sequencing are a great asset. Herein, efforts were focused on i) detecting naturally circulating viruses (Chapter 2, assay 4 and Chapter 3) and assess the potential influence of ISFs on the vector competence for RVFV transmission (Chapter 2); and ii) fully

characterize the microbiota (bacteria) of an autochthonous population of *An. atroparvus* from a former malaria endemic area of Spain (Chapter 1, assay 2).

Since RVFV has expanded its distribution range in the last decades (Hartman, 2017) and Spain showed a high suitability for RVFV occurrence in the Mediterranean region (Sanchez-Vizcaino et al., 2013), the VC of two autochthonous mosquito populations (laboratory *Cx. pipiens* and field-caught *Ae. vexans*) was assessed for the transmission of RVFV (Chapter 2). Due to the potential influence of ISVs in arbovirus transmission, in both trials, the role of an ISF was evaluated; Culex flavivirus isolated from Spanish *Cx. pipiens* captured in Huelva, Spain, in 2006, in co-infection through intrathoracic inoculations in *Cx. pipiens* (Chapter 2, assay 3), and co-infection with a naturally circulating mosquito flavivirus in field-captured *Ae. vexans* from Begues, Catalonia (Chapter 2, assay 4). Interactions between CxFV and RVFV do not seem to affect the replication of either virus, and RVFV successfully overcame midgut and salivary glands barriers. Whereas, mosquito flavivirus-RVFV interaction, although did not avoid RVFV transmission, modulated RVFV infection in *Ae. vexans*, so that, further studies on the transcriptome are needed to understand how this virus alter the viral infection within the mosquito and infer its potential use in the control of RVFV.

In the hunt for mosquito-associated viruses, the circulation of three novel putative-ISVs was detected through NGS and later by RT-PCRs in European mosquitoes (Chapter 3). For the first time *Culex*-bunyavirus/CAT (unclassified *Bunyavirales*), and Wuhan mosquito virus/CAT (family *Orthomyxoviridae*, genus *Quaranjavirus*) were identified in *Cx. pipiens*; and Alphamesonivirus/CAT (family *Mesoniviridae* genus *Alphamesonnivirus*) in *Cx. pipiens* and *Cq. richiardii* from Europe. Viral isolation attempts in mammalian and insect cells should be conducted for their better characterization. Since they were initially detected from saliva deposited and preserved in filter paper FTA cards, it is crucial to verify their host-range as they are transmissible viruses. Fact that rises concerns of their potential to

evolve from being insect-specific to dual-host viruses and become new emerging pathogens. Moreover, since the site of their detection, the Llobregat River Delta, gathers all the ecological parameters for arbovirus transmission such as WNV and Usutu viruses owing to the presence of *Culex* mosquitoes (the primary vectors), native and migratory birds (the reservoir), as well as, a highly populated area; and suitable environmental conditions for viral transmission, the potential role of these viruses in the transmission of arboviruses could be evaluated.

Furthermore, regarding the risk of malaria resurgence in European countries, the increased number of imported cases of malaria and the situation of anophelism without malaria have emphasized the necessity to update the knowledge on one of its primary vectors, *An. atroparvus*. In order to do this, a new laboratory colony (with a standard breeding protocol) of an autochthonous *An. atroparvus* population from a former malaria endemic area of Spain (Chapter 1, assay 1) is available for research purposes. Laboratory breeding produced a diversity decline as previously suggested (Rani et al., 2009; Duguma et al., 2015; Dada et al., 2020), and this might influence further VC outcomes. However, infections in early generations would provide a more reliable assessment of VC since bacterial communities in sylvan emergent specimens were similar to those in females from the second laboratory-generation (F2). In addition, finding *Serratia* and *Asaia* as part of the core microbiota of *An. atroparvus* require attention in further studies that assess the VC of this anopheline population for the transmission of most commonly imported *Plasmodium* parasites.

Finally, early detection of pathogen circulation is essential to prevent and control the spread of MBD diseases. In the last chapter of this thesis (Chapter 5), a novel approach using next generation sequencing applied on honey-baited FTA cards used in entomological surveys was validated as a suitable tool for the detection of circulating viruses in mosquitoes and the identification of the transmissible fraction of the mosquito's virome. Implementing this approach to

regular entomological and arbovirus surveillance could help preventing MBD outbreaks of known and unknown pathogenic viruses.

Overall, the study of the microbiome of vector mosquitoes contribute to a better understanding of the tripartite bacteria/virus-pathogen-mosquito interactions that may influence the vector competence of local mosquito populations and opens a path for the development of innovative approaches for more adequate vector control and pathogen surveillance strategies.

GENERAL CONCLUSIONS

1. An *Anopheles atroparvus* population from Southern Europe has been successfully established in the laboratory. This new standard colony and an updated rearing protocol are now available for malaria and arboviruses research.
2. The microbiota of *An. atroparvus* is strongly influenced by its breeding environment and by the physiology and foraging habits of the mosquitoes. It can be transstadially transmitted, from larvae to adult females, and it is partially conserved for, at least, ten generations under controlled laboratory conditions.
3. Laboratory breeding causes a microbial diversity decline in field-colonized *An. atroparvus* females. Consequently, functional and vector competence studies using field-caught and laboratory-reared specimens may provide contrasting results due to their differences in the microbiota composition. Fact that must be considered in vector research.
4. Gram-negative proteobacteria dominate the microbiota of *An. atroparvus* from the Ebro Delta. Among which, *Pseudomonas*, *Serratia*, and *Asaia* are proposed as potential candidates for the development of novel bio-control tools for European anopheles populations.
5. Catalonian *Culex pipiens* and *Aedes vexans* are competent vectors for RVFV after oral exposure under local summer conditions. Therefore, they could be involved in the transmission of RVFV in case of introduction to the European continent and should be included in surveillance and control programs for RVFV.
6. *Culex pipiens* form molestus transmits RVFV more efficiently than the hybrid form highlighting the necessity of a correct characterization of local populations of mosquito vectors to better assess the transmission risk in susceptible areas.
7. CxFV successfully infects and disseminates in Catalonian *Cx. pipiens* after intrathoracic inoculations but does not affect RVFV replication. Hence, CxFV might not alter the immune system of *Cx. pipiens* to interfere with RVFV infection, dissemination and transmission implying that CxFV might not be an efficient tool for RVFV control in these mosquitoes.
8. Naturally circulating mosquito-flavivirus in *Ae. vexans*, although did not avoid RVFV transmission, modulates RVFV infection susceptibility, although further studies are needed to confirm its

potential interference in RVFV transmission so it could be used as a bio-control tool in case of introduction of RVFV in areas where *Ae. vexans* is distributed.

9. Metagenomics applied on honey-baited FTA cards allow the detection of viruses present in the mosquitoes' saliva in field conditions demonstrating its suitability for arbovirus surveillance and for the detection of unknown and potentially pathogenic viruses. Moreover, these detections constitute the first distribution records of insect-specific viruses related to *Alphamesonivirus*, *Quaranjavirus* and unclassified *Bunyavirales* in European mosquitoes.

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