SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA17108-47439
STSM title: Pathogen detection and host-feeding patterns of field-collected mosquitoes in urban, peri-urban, rural and natural environments of Mallorca (Spain).
STSM start and end date: 30/01/2021 to 28/02/2021
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PURPOSE OF THE STSM:

Vector-borne diseases represent a public health problem worldwide. Mosquitoes can transmit several arboviruses as dengue virus, West Nile virus and other pathogens such avian malaria and filarial nematodes that cause diseases to human, domestic animals and wildlife. These diseases are currently expanding their geographical ranges. Zoonotic cases occur when one pathogen jump from natural or sylvatic environments to rural and urban areas. Thus, host-feeding patterns of mosquito species are also important to identify potential vector species. Surveillance of these diseases and the identification of potential vectors and hosts provide valuable data about the distribution and prevalence of these diseases and could be used for further implementation of disease control strategies.

The objective of the STSM project is to detect potential pathogen transmission between different environments of Mallorca Island (Spain) including the specimens collected during the harmonized surveillance of *Aedes* Invasive Mosquitoes throughout season 2020. For this, a detection of pathogens and host-feeding preferences in different species of mosquitoes was performed during the stay at the Bernhard Nocht Institute for tropical medicine.

A total of 2,461 mosquitoes were collected weekly between July and November 2020 in ten sample sites including urban areas (three sites), peri-urban areas (including the ones used for the AIMSurv) (four sites), rural areas (livestock farms) (three sites), natural conservation areas (one site) and dog pounds (one site).

Samples were collected by four different adult traps per sampling site: one BG-Sentinel, one CDC baited with CO_2, one CDC with light and one Onderstepoort with UV light. Traps were set from two hours before dusk to two hours after down.

Mosquitioes were killed at -20ºC and determined on a chill table. Female mosquitoes were kept at -80ºC in 1.5 ml Eppendorf tube pools of 25 individuals while blood-engorged females were individually kept -80ºC in 1.5 ml Eppendorf tubes.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

1. Homogenisation of blood-fed specimens and host detection.

A total of 77 blood-fed (BF) specimens were first classified according to Sella score following Detinova *et al*. (1962). These specimens comprised four *Aedes albopictus*, one *Aedes caspius*, one *Culiseta*

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longiareolata and 71 *Culex pipiens* s.l. Then, specimens were homogenized individually using zirconia beads and 500 µl of DMEM (500 ml DMEM High Glucose, 5 ml PenStrep and 5 ml Amphotericin B). Homogenisation was performed in a TissueLyser at 30 Hz (TissueLyser II) for 3 min.

Before RNA/DANA extraction, samples were centrifuged for 1 min. at 8.000 rpm at 4°C. 140 µl of the supernatant were used for extraction and 300 µl for long-term storage. RNA/DNA was extracted using QIAamp Viral RNA Kit (Qiagen, Hilden, Germany).

For host detection, each BF individual was screened with two PCR with different sensitivity for mammals, birds; cytochrome b or 16S rRNA gene: Burkett-Cadena *et al*. (2008) modified from Kitano (2007) and Kocher *et al*. (1989). PCR amplificates were sent for sequencing and sequences analysed by blasting the NCBI genbank

2. Homogenisation of non-BF specimens.

A total of 206 pools of mosquito females from nine different species (*Ae. albopictus*, *Ae. caspius*, *Aedes detritus*, *Aedes mariae*, *Cs. longiareolata*, *Culex laticinctus*, *Culex perexiguus*, *Cx. pipiens* s.l. *and Culex theileri*) were homogenized using zirconia beads and 500 µl of DMEM (500 ml DMEM High Glucose, 5 ml PenStrep and 5 ml Amphotericin B). Homogenisation was performed in a TissueLyser at 30 Hz (TissueLyser II) for 3 min.

Before RNA/DNA extraction, samples were centrifuged for 1 min. at 8.000 rpm at 4°C. 140 µl of the supernatant were used for extraction and 300 µl for long-term storage. RNA/DNA was extracted with a KingFisher Flex 96 Deep-Well Magnetic Particle Processor using the MagMAX CORE Nucleic Acid Purification Kit.

3. Identification of *Culex pipiens* s.l. specimens

Typing of BF and non-BF Culex pipiens individuals were performed by qPCR following the Culex-taxo-PCR protocol (Rudolf et al. 2013) to differentiate the *Cx. pipiens* biotypes: *pipiens*, *molestus* and/or *torrentium*.

4. Pathogens screening

Samples of BF and non-BF mosquitoes were tested for different pathogens using established protocols. For the most important arboviruses in Europe we use a pan-flavivirus (Flaviviridae NS5, modified from Chao *et al*. (2007)) and a pan-alphavirus (Alpha nsP4, Eshoo *et al*. (2007)) RT-PCR.

Screening for avian malaria was performed with avian malaria Haemoproteus and Plasmodium/Leucocytozoon nested PCR (Bell et al. 2015) and screening for *Dirofilaria repens* and *D. immitis* were conducted with a qPCR following the Dirofilarien-PCR protocol (Sulesco et al. 2016).

PCR amplificates were sent for sequencing and sequences analysed by blasting the *NCBI genbank.*

DESCRIPTION OF THE MAIN RESULTS OBTAINED

1. Host detection of BF specimens

A total of 72 BF individuals were able to detect hosts with Burkett-Cadena *et al*. (2008), showing that the 93.1 % of individuals fed on mammals and 6.9 % fed on birds. The PCR performed with Kocher *et al*. (1989) determine de the hosts of 61 BF mosquitoes, being 26.2 % mammals and 73.8 % birds. The negative host detections may be related to low or high Sella score.

Results showed that *Ae. albopictus* collected in peri-urban areas fed on cats (*Felis sylvestris*) and rats (*Rattus rattus*), whereas *Ae. albopictus* from urban areas fed on human (*Homo sapiens*).

The single *Ae. caspius* individual collected in a peri-urban area fed on rabbits (*Oryctolagus cuniculus*) and robins (*Erithacus rubecula*) and the single *Cs. longiareolata* from the dog pound fed on human (*H. sapiens*).

The species *Cx. pipiens* s.l. fed on mammals such as human (*H. sapiens*), dogs (*Canis lupus familiaris*),

cats (*Felis catus*), cattle (*Bos* sp.), pigs (*Sus scrofa*) and a wide range of birds including: chickens (*Gallus gallus*), Eurasian hoopoes (*Upupa epops*), doves (*Columba livia*), stone-curlews (*Burhinus* sp.), robins, blackbirds (*Turdus* sp.), etc.

2. Identification of *Culex pipiens* s.l. biotypes

The 71 BF *Cx. pipiens* s.l. individuals were identified as *Cx. pipens molestus* (65%), hybrids (*Cx. pipiens pipiens* x *molestus*) (21%) and *Cx. pipiens pipiens* (14%). Conversely, 103 pools of non-BF *Cx. pipiens* s.l. females were identified hybrids (83 %), *Cx. pip. molestus* (11%), *Cx. pip. pipiens* (5%) and 1% of false positives.

3. Identification of pathogens

The pan-alphavirus RT-PCR was negative for all samples. 39 BF individuals and nine pools of non-BF females showed RNA amplification after the pan-flavivirus RT-PCR. Regarding the avian malaria, 60 BF individuals and 53 pools of non-NF females showed DNA amplification after the Haemoproteus nested PCR. In addition, 16 BF individuals and 32 pools of non-BF females also showed DN amplification after the Plasmodium/Leucocytozoon nested PCR. These samples were sent for sequencing and results are showed in table 1 and 2:

Table 1. Pathogens recorded in BF individuals

Species	Habitat	Host/s	Pathogen
Ae. albopictus	Peri-urban	*R. rattus*	*Plasmodium* sp. cluster D
Ae. caspius	Peri-urban	*O. cuniculus* and *E. rubecula*	*D. repens*
Cx. pipiens molestus	Peri-urban	*H. sapiens* and *Turdus merula*	*Plasmodium* sp. HMA-2012
Cx. pipiens molestus	Peri-urban	*H. sapiens* and *Curruca melanocephala*	*Plasmodium* sp. AP66
Cx. pipiens molestus	Peri-urban	*H. sapiens* and *Bos taurus*	*Plasmodium* sp Plascoq8
Cx. pipiens molestus	Rural	*H. sapiens*	*Plasmodium* sp. B1
Cx. pipiens molestus	Dog pound	*H. sapiens* and *C. livia*	*Haemoproteus* sp. HaemK1
Cx. pipiens hybrid	Peri-urban	*H. sapiens* and *Sylvia atricapilla*	*Plasmodium relictum*

Table 2. Pathogens recorded in pools of non-BF females

Species pool	Habitat	Pathogen
Ae. albopictus	Peri-urban	WNV
Ae. albopictus	Dog pound	*Plasmodium* sp. NG1769P
Cx. pipiens molestus	Peri-urban	*Plasmodium* sp.
Cx. pipiens hybrid	Dog pound	*P. relictum*
Cx. pipiens hybrid	Dog pound	*P. relictum*
Cx. pipiens hybrid	Dog pound	*Plasmodium* sp.
Cx. pipiens hybrid	Dog pound	*Hemoproteus columbae*
Cx. pipiens hybrid	Rural	*Plasmodium* sp. U12
Cx. pipiens hybrid	Rural	*Plasmodium* sp. PlasCoq3.
Cx. pipiens hybrid	Urban	*Plasmodium* sp. S33

FUTURE COLLABORATIONS (if applicable)

We start collaboration between the Applied Zoology and Animal conservation research group and the BNITM. 418 mosquitoes from three different species (*Ae. albopictus*, *Ae. caspius* and *Cx. pipiens* s.l.) collected in nine different sites of Mallorca Island were kept for wing morphometry analysis in the BNITM lead by Dr. Renke Lühken. In addition, a local project in the Balearic islands entitled *Prevalence of arboviruses and other pathogens transmitted by dipterans in livestock farms of the Balearic islands* has been already submitted within a project call from the government of the Balearic Islands.

The detection of pathogens and hosts-feeding patterns in mosquitoes collected in different environments could contribute in surveillance programs for invasive species as *Ae. albopictus* and improve control intervention strategies against *Aedes*-borne diseases contributing in this way to the scientific objectives of the AIM-COST.