STSM SCIENTIFIC REPORT

STSM GRANTEE: Mgr. Kristyna Hlavackova

COST Action: TD1303

STSM title: Analysis of population dynamics of pre-alpine biting midges (Diptera: Ceratopogonidae) by MALDI-TOF mass spectrometry and vector capacity traits

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Location: Institute of Parasitology, University of Zurich, Zurich, Switzerland.

Host: Institute of Parasitology, National Centre for Vector Entomology, University of Zurich, Switzerland (Dr. Eva Veronesi, Prof. Dr. Alexander Mathis).

Purpose of the STSM

My STSM took place at the Swiss National Centre for Vector Entomology, a leading research institution for species identification of medically significant insects by protein profiling using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) . The aim of my STSM was to widen my knowledge about sample preparation for MALDI-TOF MS analysis, as well as about the evaluation of the results. Along with that I was to be trained on the morphological identification of Ceratopogonidae, their bionomics and monitoring methods. *Culicoides* biting midges belong to the most important vectors of pathogens of livestock. They are especially involved in the transmission of orbiviruses, of which the most important ones are bluetongue virus that affects mostly livestock and African horse sickness virus which can be lethal for horses. Moreover, I was also involved in a Simuliidae project focusing on establishing a protein database for a straightforward MALDI-TOF MS identification. This project involved training in trapping and morphological identification of Simuliidae, as well as DNA-barcoding and MALDI-TOF MS analyses.

Description of the work carried out during the STSM

The project I was involved in focused on vector capacity of (pre-)alpine midges for orbiviruses as part of a Veterinary doctoral thesis that is ongoing at the host institution (UZH). My activities were done accordingly to the working plan.

- **Population dynamics of pre-alpine *Culicoides* species (Diptera: Ceratopogonidae).**

I attended field trips to the pre-alpine regions of Davos and Lenzerheide (canton of Grisons, Switzerland) where collections of *Culicoides* were carried out. For adult collections, standard Onderstepoort black-light suction traps (OVI traps) equipped with UV-light tubes were activated. Traps were operated overnight starting approx. two hours before sunset until 2 hours after sunrise the day after. Seven farms were chosen for the population dynamic studies. Field caught midges were then identified down to group/species level using both morphological techniques and molecular analysis by MALDI-TOF MS. Samples for MALDI-TOF MS analysis were prepared according to a standardized protocol: only thoraxes were used for protein extraction in 10 µl of 25% formic acid, tissue homogenization was performed using pestles. Protein homogenates were premixed with SA-matrix, spotted on steel MALDI plates and let to co-crystalize. Protein spectra were obtained on a MALDI-TOF Mass Spectrometry Axima Confidence Machine and analyzed with SARAMIS Premium software.

- **Bionomics of pre-alpine *Culicoides*.**
  - Sugar feeding behavior

Wild collected *Culicoides* were tested for fructose presence using the cold anthrone test. A sub-sample of dead and alive *Culicoides* from OVI traps were placed immediately after collecting on dry ice and then stored at -80 °C until fructose/sucrose test examination. All specimens were first morphologically identified at group level (Obsoletus, Pulicaris group, others) using a stereomicroscope, and they were also defined as nulliparous/parous/ blood fed/gravid. Each specimen was placed in a separate tube and dropped in a chloroform:methanol (1:1) mixture to remove the wax layer in order to avoid mechanical homogenization with pestles, as this procedure was found to be less sensitive in previous experiments run with *Culicoides* trapped at UZH campus. Afterwards, an anthrone reagent (0,2 ml /sample) was added to the tube, and after one hour the samples were checked for color change from yellowish to light or dark blue.

  - Characterization of breeding sites

In parallel with the collection of live midges, I was also involved on the characterization of breeding sites for *Culicoides*. At each farm where adult traps were activated soils samples were collected at different points and from each point a total of 3 samples were collected, 50 cm apar from each other. All the soil samples were allocated into mini insect breeder boxes (bugdorm.com) that functioned as emergence traps, allowing emerged insects to be collected on a small netted container allocated on the top of the box. All the boxes were stored at approximately 24 °C and checked weekly for emerging *Culicoides*.

Every week the insects that emerged from the soil were transferred to 70% ethanol for further examination under a stereomicroscope.

- **Evaluation of MALDI-TOF MS analysis for Simuliidae species identification.**

Species of the family Simuliidae were collected in the region Thurauen, Davos and Lenzerheide in Switzerland, and some specimens from Serbia were available (kindly donated by Aleksandra Cupina from Novi-Sad University). Collections were performed using CDC light traps operated overnight; specimens were stored on dry ice and later dried at -20 °C or in 70% ethanol at 4 °C. All specimens were first identified under the stereomicroscope according to their morphology using different identification keys. From selected samples DNA was isolated using a commercially available Kit (Qiagen) following the protocol for DNA isolation from insects. Using primers LepF1 and LepR1 approximately 600 bp of the mitochondrial cytochrome oxidase I gene was amplified by PCR. The obtained amplicons were visualized under UV on agarose gel electrophoresis, were purified using Qiagen Kit and sequenced directly. Obtained sequences were compared to those downloaded from GenBank and BOLD database using BLASTn.

For DNA isolation only the first four abdominal segments were used, thoraxes were later used for MALDI-TOF MS analysis performed according to the same protocol described above.

Description of the main results obtained

As my STSM was deferred one month and is not yet finished, all my results are just partial.

- **Population dynamics of pre-alpine *Culicoides* species (Diptera: Ceratopogonidae).**

This part of the study is still on-going and so far only very preliminary results are available. The amount of midges collected was strongly dependent on weather conditions, lower night temperatures (approx. 6 °C) decreased the quantity of insects collected, especially of *Culicoides* (about 50 midges per trap); whereas during warmer nights (approx. 12 °C) the numbers of caught *Culicoides* was about 1000 midges per trap. According to the pre-sorting of *Culicoides* to group level, species of the Pulicaris group seem to be more prevalent in the Davos region, while in Lenzerheide there were more specimens belonging to the Obsoletus group.

- **Bionomics of pre-alpine *Culicoides*.**
  - Sugar feeding behavior

In the first experiment the sensitivity of the anthrone test was evaluated using *Culicoides* caught at the UZH campus. The usage of chloroform:methanol mixture was found to be more efficient

rather than crashing midges by pestles. No difference was observed between *Culicoides* killed immediately after catching and those killed the day after (all *Culicoides* were killed at -20 °C).

Further, only *Culicoides* from the region Davos and Lenzerheide were tested. In total 620 of midges were sorted for the anthrone test so far and 62 of *Culicoides* had to be excluded from testing because either their abdomen or decisive characters were missing. Results are presented in table 1.

Table 1: Percentage of fructose-positive *Culicoides* females caught at farms in Davos and Lenzerheide area (canton of Grisons, Switzerland).
*in bracket, absolute number of females fed on fructose/total female tested

site	GRO5-15-7-2	**total**	55% (11/20)	
group	**nulliparous**	**parous**	**blood-fed**	**total**
Obsoletus	35% (7/9)	10% (2/6)	0	45% (9/15)
Pulicaris	5% (1/2)	5% (1/3)	0	10% (2/5)

site	GRO7-15-7-2	**total**	63,6% (161/253)	
group	**nulliparous**	**parous**	**blood-fed**	**total**
Obsoletus	15,5% (40/55)	4,6% (12/25)	0,8% (2/2)	20,9% (54/82)
Pulicaris	26,6% (67/92)	15,8% (40/75)	0,3% (1/4)	42,7% (108/171)

site	GRO3-15-7-17	**total**	61,1% (62/101)	
group	**nulliparous**	**parous**	**blood-fed**	**total**
Obsoletus	12,8% (13/21)	10,9% (11/17)	0	23,7% (24/38)
Pulicaris	24,6% (25/38)	12,8% (13/25)	0	37,4% (38/63)

site	GRO4-15-7-17	**total**	59,9% (121/202)	
group	**nulliparous**	**parous**	**blood-fed**	**total**
Obsoletus	24,7% (50/71)	12,9% (26/50)	0	37,6% (76/121)
Pulicaris	14,9% (30/50)	7,4% (15/31)	0	22,3% (45/81)

site	GRO5-15-7-17	**total**	37,2% (16/43)	
group	**nulliparous**	**parous**	**blood-fed**	**total**
Obsoletus	11,6% (5/16)	11,6% (5/12)	0	23,2% (10/28)
Pulicaris	11,6% (5/6)	2,4% (1/9)	0	14% (6/15)

My results indicate that the percentages of sugar-feeding pre-alpine midges are lower than those identified in a previous study with midges from the Swiss lowlands (Kaufmann *et al.* 2014).

- Characterization of breeding sites

Samples were collected at seven different farms in the region of Davos and Lenzerheide. Several sample points were identified using the following criteria:

1) fresh (few days old) manure/faces
2) outdoor old (1 year old) and composted manure
3) indoor cowshed
4) muddy area around animal barns or near to old manure where standing wet soil is present
5) composted soil
6) wet soil around water tanks
7) wet soil close to manure
8) less wet soil close to manure
9) mixed manure and moist organic matter

Out of 93 soil samples collected during June and July, none was found positive for specimens of the *Culicoides* genus. Samples were checked weekly under a stereo microscope.

Complete absence of emerging insects was observed in six soil samples (collected at two different farms) until 4 weeks post collection. The most abundant insects emerging from collected soil samples were members of the families Muscidae, Psychodidae, Sciaridae and Chamaemyiidae (identified according to their morphology).

Possible explanations are: the chosen sites are no midges breeding sites, pre-alpine midges have only one generation per year, predation of larvae from other insects or the developing time is longer hence they still have to emerge.

- **Evaluation of MALDI-TOF MS analysis for Simuliidae species identification.**

Trapped black flies were first identified by morphology (n=~300) at least to subgenus level (results in table 2 below).

Table 2: Overview of specimens of the family Simuliidae identified according to their morphology at least to subgenus level.
*nd: not determined

SITE	TUBE	SUBGENUS	SPECIES
DAVOS LENZERHEIDE	GRO2-15-7-9	*Wilhelmia*	nd
	GRO4-15-7-9	*Eusimulium*	nd
	GRO2-15-7-16	*Prosimulim*	nd
	GRO5-15-7-16	*Eusimulium*	nd

SITE	TUBE	SUBGEBUS	SPECIES
THURAUEN	T01/30-4-15	*Wilhelmia*	*equinum*
	T02/30-4-15	*Wilhelmia*	*equinum*
	T03/30-4-15	*Wilhelmia*	*equinum*
	T04/30-4-15	*Wilhelmia*	*equinum*
	T05/30-4-15	*Wilhelmia*	*equinum*
	T07/30-4-15	*Wilhelmia*	*equinum*
	T08/30-4-15	*Wilhelmia*	*equinum*
	T09/30-4-15	*Wilhelmia*	*equinum*
	T10/30-4-15	*Wilhelmia*	*equinum*
	T06/18-5-15	*Wilhelmia*	*equinum*
	T05/4-6-15	*Wilhelmia*	*equinum*

SITE	TUBES	SUBGENUS	SPECIES
SERBIA	1-4,16,21	*Boophthora*	*erytrocephalum*
	6-10,17	*Wilhelmia*	*balcanicum*
	11-12	*Simulium*	ornatum complex

DNA was isolated from 30 randomly chosen specimens belonging to the family Simuliidae (see the table 3 below). PCR was performed with all these specimens, and sequences could be obtained from 27. Three specimens did not yield an amplicon; therefore the DNA concentration was measured by NanoDrop. The DNA concentration of all 3 samples was approx. 100 ng/µl, hence an inhibition test was applied, but was negative. DNA of these samples was then visualized on agarose gel to reveal possible fragmentation, which was not confirmed. Lastly the PCR reaction was repeated using a new pair of primers (LCO1490, HCO2198) which target a slightly different sequence, but amplify the same region of the COI gene. This PCR was successful and samples were sent for sequencing.

Obtained sequences were analyzed by using BLASTn and paired to GenBank sequences for species identification. In case of the subgenus *Wilhelmia* the identification was problematic, for the species of this subgenus are morphologically but also sequentially (COI) very similar. In this case (*S. lineatum*) I would think the morphological identification is more plausible than the identification acquired by DNA sequence.

Table 3: Overview of the different methods applied for individual samples of the family Simuliidae in order to identify them.

ORIGINAL TUBE	SAMPLE NAME	MORPHOLOGY	SEQ	MALDI-TOF	SPECIES	ACCESSION NO.
T06/18-5-15	T06_1	*Wilhelmia*	COI	DONE	*S.equinum*	GU203457.1
	T06_2	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T06_3	*Wilhelmia*	COI	DONE	*S.equinum*	GU203457.1
T05/4-6-15	T05_4	*Wilhelmia*	COI	DONE	*S.equinum*	GU203461.1
	T05_5	*Wilhelmia*	COI	DONE	*S.equinum*	GU203457.1
	T05_6	*Wilhelmia*	COI	DONE	*S.equinum*	GU203457.1
T04/30-4-15	T04_7	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T04_8	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T04_9	*Wilhelmia*	COI	DONE	*S.equinum*	GU203461.1
T07/30-4-15	T07_10	*Wilhelmia*	COI	DONE	*S.equinum*	GU203457.1
	T07_11	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T07_12	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
T03/30-4-15	T03_13	*Wilhelmia*	COI	DONE	*S.equinum*	GU203461.1
	T03_14	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T03_15	*Wilhelmia*	COI	DONE	*S.equinum*	GU203461.1
T01/30-4-15	T01_16	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T01_17	*Wilhelmia*	COI	DONE	*S.equinum*	GU203464.1
	T01_18	*Wilhelmia*	COI	DONE	*S.equinum*	GU203461.1
GRO2-15-7-9	GRO2_1	*S.equinum*	COI	X	*S.equinum*	GU203464.1
GRO4-15-7-9	GRO4_2	*Eusimulim*	COI	X	*S.vernum*	GU072981.1
	GRO4_3	*Eusimulim*	COI	X	*S.crenobium*	GU072930.1
SERBIA-tube 1	SERB_4	*B.erythrocephlaum*	COI	X	*S.erythrocephlaum*	KF640028.1
	SERB_5	*B.erythrocephlaum*	COI	X	*S.erythrocephlaum*	KF640028.1
	SERB_6	*B.erythrocephlaum*	COI	X	*S.erythrocephlaum*	KF640028.1
SERBIA-tube 9	SERB_7	*W.balcanicum*	COI	X	*S.lineatum*	KF990255.1
	SERB_8	*W.balcanicum*	COI	X	*S.balcanicum*	KF990258.1
	SERB_9	*W.balcanicum*	COI	X	*S.lineatum*	KF990254.1

From 6 other samples DNA was isolated as well as PCR ran, but I have not obtained the sequences yet. Also the rest of the listed samples were processed by MALDI-TOF MS analysis, but as well the results are not finished yet.

Future collaboration

Amongst all the aspects that were investigated by Kristyna, the evaluation of the use of MALDI-TOF MS approach for Simuliidae identification was indeed the most innovative and novel one as this was the first time that MALDI-TOF MS was applied for Simuliidae. Knowing the difficulties on molecular and morphological approaches, if we can find a method that will increase the

sensitivity of the test and hence the reliability of the species determination, it would be a gold standard for further approaches.

Indeed two months were not enough to validate MALDI-TOF MS tests for Simuliidae identification. The preliminary data here produced by Kristyna (more still to come which are still on going by the time this manuscript was written) will be definitely taken over with more analyses.

According to her future plans and availability, we are more than keen to keep the contact with her and her Institution in consideration of potential opportunities for further collaboration on this subject.

Foreseen publication

The preliminary data here produced will serve for future investigation and hopefully we will be able to use them for publication in a peer reviewed journal. Kristyna's contribution will obviously be acknowledged as co-author on the publication/s which is/are pertinent to this subject and to the material she has produced during her stay at our Institution.

Conformation by the host institution of the successful execution of the STSM

Kristyna has worked very intensively with a rich detailed programme of activities most of them completely new for her. Her project was focussed on two groups of vectors: *Culicoides* (Diptera: Ceratopogonidae) and Simuliidae. She investigated ecological aspects of the two including breeding sites characterization, sugar feeding behaviour, and identification methods using morphological and molecular approaches highlighting the limitation and advantages of the two.

Considering the short time of her visit (8 weeks), she indeed worked very hard and managed to get also good original data.

Kristyna has shown strong initiative and independency during her stay at the IPZ. She has indeed a very good scientific approach and scientific thinking, with good flexibility to modify the plans and to test several methods in alternative to the one that do not work and she is very well organized. She was also very keen to work long hours which is an excellent approach when working with seasonal activities and live material such as insects.

She has been working hard also on literature review and discussing papers with the IPZ staff showing good critical approach. Her molecular background knowledge on sand flies was indeed useful and helped her to apply similar techniques during her stay here.

Kristyna also had the opportunity to visit Mabritec Company, a specialized centre for identification of microorganisms and characterization of biological systems by mass spectrometry. Her visit was very successful, she could also be trained directly from the Mabritec personnel on MALDI-TOF MS and she enjoyed very much and she was very enthusiastic about exchange knowledge with the staff.