SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

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

Action number: CA18221
STSM title: Evaluation of the influence of two widely used pesticides on *Bufo viridis* tadpoles
STSM start and end date: 15/03/2021 to 23/03/2021
Grantee name: Bruno Bekić

PURPOSE OF THE STSM:

One of the main reasons why I apply for STSM is the lack of adequate equipment in the home institution to perform these measurements. I also believe that this experience will help me in my future education and employment and that I will be introduced to new techniques and new laboratory equipment important when conducting research.
Stress biomarker analysis will be carried out on tissue samples obtained from the toxicity study of the influence of Alpha cypermethrin and Difenoconazole on *Bufo viridis* tadpoles. Current data set compiles LD50 values and experimental behavioural tests, that we want to complement with the analysis of three biochemical markers of stress: activity of catalase, protein carbonyl content and estimated potential maximum capacity for metabolism (ETS). Obtained data will provide better insight into the sensitivity of green toads to pesticides, since this species is commonly present in the vicinity of agricultural land, and has a great exposure risk. Researcher on the STSM would conduct biochemical analysis on the existing samples and under supervision in the host laboratory.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

First day hosts toured me around the facilities and showed all the labolatories and instructions to use equipment. We planned the work of the following days and started by marking and weighing Eppendorf tubes for measurements. Over the following days, all the measurements were done in laboratory. The last day I worked at the computer to analyse the data and below is a brief description of the measurements performed.

During this research, 3 biochemical protocols were used to obtain measurements of stress biomarkers (catalase activity, protein carbonyl content and electronic transport system activity (ETS) at three different temperatures) on *Bufo viridis* tadpoles. The protocol to assess electronic transport system activity (ETS) followed the methodology described in Žagar et al. (2015). In the last step, the microtiter plates were incubated at 3 different temperatures (20°C, 24°C and 28°C) for 15 minutes. After incubation, 50 µL of quenching solution (formaldehyde and H_3PO_4) was added and the absorbance was measured at 490 nm on a SynergyMX microplate reader. From the measured values, we calculate the concentration of oxygen consumed, ie the ETS activity that corresponds to the amount of formazan formed by the decrease in INT (G.-Tóth et al., 1995).

Determination of the presence of carbonyl groups was done done following the protocol described in Li-Byarlay et al. (2016). In the last step, 100 µL of each sample was transferred to microtiter plates. Absorbance was then measured at 375 nm on a SynergyMX microplate reader. After that 5 µL of each sample was

transfered to another set of wells to measure the amount of protein per sample to be able to calculate the carbonyl protein content.

Catalase activity was estimated using the protocol decribed in Aebi (1984). The final mixture was poured into a quartz cuvette and the decrease in catalase activity was measured at 240 nm for 2 min every 30 seconds at 25°C on a PerkinElmer Lambda 25 UV / VIS spectrometer to estimate the antioxidant enzyme activity of catalase (antioxidant capacity).

Literature sources:

Aebi, H. 1984. Catalase in vitro. Methods in enzymology, Elsevier. 105, 121-126.
G.-Tóth, L., Szabo, M., Webb, D.J. (1995) Adaptation of the tetrazolium reduction test for the measurement of the electron transport system (ETS) activity during embryonic development of medaka. Journal of fish biology, 46, 835-844.
Li-Byarlay, H., et al. 2016. Honey bee (Apis mellifera) drones survive oxidative stress due to increased tolerance instead of avoidance or repair of oxidative damage. Exp. Gerontol. 83, 15-21.
Žagar, A., Simčič, T., Carretero, M.A., Vrezec, A. (2015) The role of metabolism in understanding the altitudinal segregation pattern of two potentially interacting lizards. Comp. Biochem. Physiol. Part A: Molecular & Integrative Physiology, 179, 1-6.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

All our labolatory analysis were successful and during the STSM stay we also already made some preliminary results that are summarized bellow.

Altogether, we had 62 samples from 3 different experiments. The first experiment was using pesticide Alpha cypermethrin and the pesticide concentrations were 0, 0.1, 0.2, 0.4, 0.6, 0.6, 1, 2, 3, 4, and 5 µg/L. The results of measuring ETS activity showed that there are no excessive deviations and that there are values that are higher and lower than the control and that there is no difference in activity at different temperatures (20, 24 and 28°C). Further, catalase activity measurements showed increased activity at lower concentrations and that at these concentrations catalase is active in suppressing oxidative stress, while at higher concentrations of pesticides it is impossible to degrade the formed hydrogen peroxide and that there is an increase in oxidative stress. The results of carbonyl measurements showed increased values at all concentrations compared to the control. As it was found that at low concentrations of pesticides, catalase manages to suppress oxidative stress by degrading hydrogen peroxide, but at higher concentrations its action ceases and decreases and this is manifested by increases in the amount of carbonyl, which is actually a sign of protein damage.

In the second experiment, the pesticide Difenoconazole was used at concentrations of 0, 0.01, 0.02, 0.04, 0.06, 0.08 and 1 mg/L. The results of measuring ETS activity showed that, as in the previous experiment, there were no excessive differences between concentrations and different temperatures. Catalase activity is reduced even at low concentrations, which indicates a marked harmfulness of this pesticide, while the values of carbonyl are higher than control at the lowest pesticide concentrations, which indicates that Difenoconazole is a more harmful pesticide than Alpha cypermethrin and that protein damage occurs at very low concentrations.

The same pesticide alpha cypermethrin was used in the last experiment, but at similar and higher concentrations of 0, 0.2, 1, 2.5, 5, 7.5 and 10 µg/L. The results of measuring ETS activity showed that the metabolic apparatus was not so damaged, but there is an increased metabolic activity at lower concentrations of pesticides, which can be associated with catalase whose activity is increased at lower concentrations, which prevents cell damage, while at higher concentrations, catalase cannot decompose hydrogen peroxide formed by the action of pesticides and there is an increase in oxidative stress, which is also manifested in a decrease in ETS activity. The amount of carbonyl increases due to protein damage caused by oxidative stress caused by the pesticide Alpha cypermethrin.

FUTURE COLLABORATIONS (if applicable)

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