Integrated assessment of chemical pollution and its impacts on wild populations of *Crocodylus moreletii* in the Mexican Yucatan Peninsula

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Purposes

The proposed study will measure levels of metals and trace elements (V, Cr, Mn, Co, Cu, Zn, Se, As, Rb, Sr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, Hg, Tl, Pb, and Bi) and POPs (e.g., PCBs, dichlorodiphenyltrichloroethane (DDTs), poly-brominated diphenyl ethers (PBDEs), and perfluoroalkyl substances (PFASs)) in blood plasma, caudal scutes, and claw samples of 11 wild *C. moreletii* populations within the YP (n = 10 per site).

Simultaneously, we will also reveal transcriptome (~10,000 transcripts) and metabolome profiles (hundreds of lipids, carbohydrates, amino acids, and hormones) in these samples. Bioinformatic approaches will be applied to determine the biomarkers and the biological pathways/networks affected by contaminants **[1]**.

Materials and Methods

Our proposed plan is being conducted in two teams: Fieldwork in Mexico and Lab work in Japan. For the fieldwork in Mexico, crocodile capture and collection of samples was executed following our well-established protocols, targeting reproductive adults (>1.0 m total length) **[2, 3]**. Blood samples (whole and plasma) were drawn from all captured individuals prior to their body size measurement and then caudal scutes and claws were obtained **[2, 3]**. To determine health condition, blood smears were prepared for blood cell counts. Eight fieldwork campaigns for sample collection were conducted from July 2021 – July 2023. The previous plan only envisioned six campaigns from July 2021 – November 2022; however, the number of samples was below 70%. Thus, two

more campaigns were conducted in 2023 to achieve the number of samples expected, and simultaneously, a postdoctoral collaboration from a researcher from Northern Mexico allowed not only to achieve the targeted number but to get 12% more samples than expected in the original plan. Blood counts in Mexico are still in progress.

Samples were preserved frozen, and transcriptome samples were preserved in RNAlater® solution (Invitrogen™) until their arrival at the laboratory of the Autonomous University of Campeche (CEDESU). The second batch of samples exportation permission was obtained in August 2024, and samples were received in the host researcher's laboratory at Ehime University, Japan in January 2025.

For the Lab work in Japan, the first batch of samples of blood samples, claws and scutes were digested in a microwave system, and metals and trace elements were determined by HG-AAS, ICP-MS, and CV-AAS **[4]**. POPs in blood plasma and scutes were determined by GC-MS **[1]**. Transcriptome profiles in soft tissues of caudal scutes were successfully determined by next-generation RNA sequencing (RNA-seq) using Illumina HiSeq 2500 system **[1].** Metabolites will be determined in blood plasma by LC coupled with Q-TOF-MS **[5-7]**. Differences in contaminant concentrations between populations will be tested through left-censored data analyses **[8]**, followed by post-hoc analyses. Relationships between contaminant levels, metabolites and transcriptome profiles will be evaluated using class coinertia analysis (CIA) and factor analysis for multiple testing (FAMT) **[1]**. For mRNAs and metabolites that exhibit relationships with contaminant levels, enrichment analysis of transcription factors, pathways, networks, and diseases will be performed using databases (KEGG, Reactome and DiseaseComps) and bioinformatics analysis tools (DAVID, STRING, Cytoscape and TfactS).

Results

By July 2023, a total of 125 individuals were captured and 858 samples were obtained for this project. We observed 29.5% of the captured crocodiles are above their "optimal" weight, while 70.4% of them are below their "optimal" weight. Metal and POPs concentrations have been determined in the first batch of samples (fig. 1) and 20 libraries of good quality for transcriptome analysis were successfully generated. Currently we are processing the transcripts by two bioinformatic approaches: mapping genes by using a genome reference (*Alligator misssissippiensis*) or *de novo* analysis to generate our mRNA reference based

on our libraries results. A careful analysis of the methods for the mappings using the genome reference is being conducted, as we are comparing two algorithms and three different pipelines (two command-based lines and one interface software). Also, preliminary results after processing data using one of the command-based pipelines have been presented. Subsequent analysis of data with mixOmics for biomarker discovery, comparing four sites, sexes and site classification have been conducted (figs. 2-4). We are still conducting tests, and we pretend to extract RNA from the new batch of samples, conduct the same analysis and enhance the presented models and compare more sites, including highly disturbed environments, such as urban lakes. We will also perform IPA analysis to understand the pathways affected by the contaminants and combine them with metabolome results.

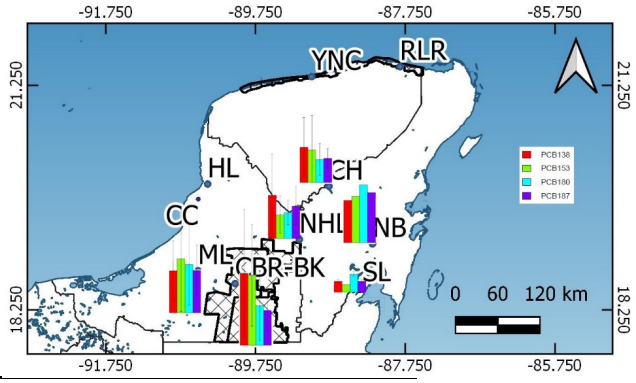

Figure 1. PCBs concentration profiles in blood plasma of *C. moreletii* samples.

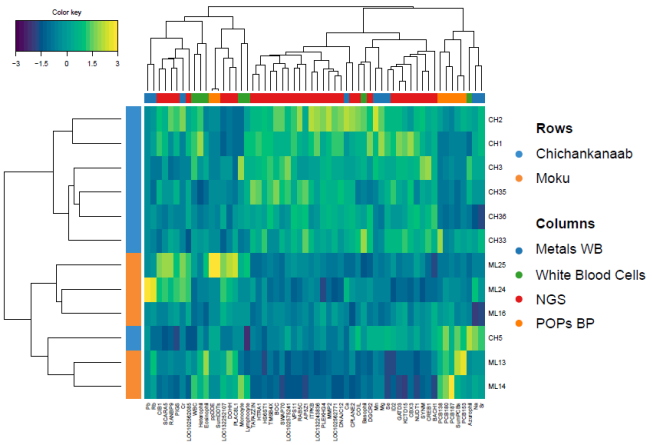

Figure 2. Heatmap analysis of 4 datasets for *C. moreletii* in two sites, by mixOmics.

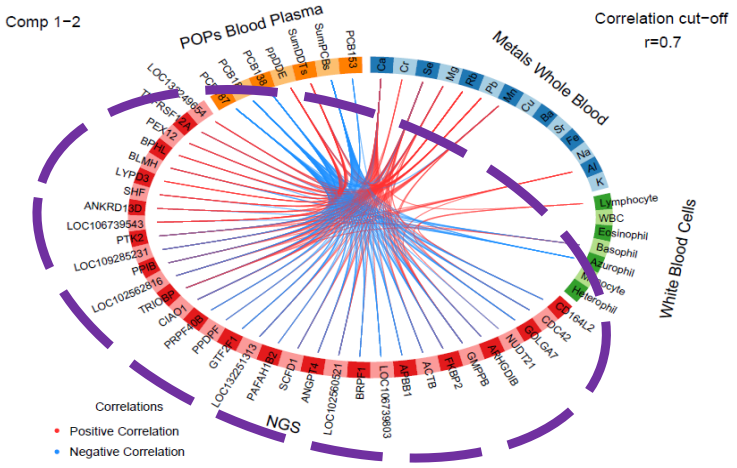

Figure 3. Circos plot by mixOmics integrating four datasets for discovery of potential biomarkers for *C. moreletii*.

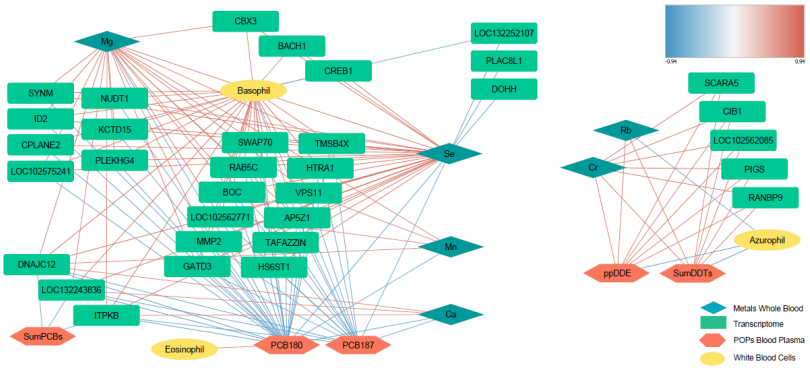

Figure 4. Integration of four datasets for networks of contaminants, white blood cells, and gene expression for *C. moreletii*.

Future Challenges

1) We expected to receive 585 samples from the second batch this year. However, bureaucratic issues, we only managed to bring a batch of 387 samples in Japan. Thus, we will aim to bring the last batch of approximately 198 samples remaining in Mexico whenever possible.

2) We still need to consider enough budget to generate 60 libraries of RNA, based on the number of samples collected and received for transcriptome.

3) After getting the best bioinformatics approach for the mappings, we need to select the best approach for the integration of all the datasets.

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