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 is currently under issuance for their subsequent exportation to the host researcher's laboratory at Ehime University, Japan.

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 (figs. 1 and 2) and 16 libraries of good quality for transcriptome analysis were successfully generated (figs. 3 and 4). 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. Depending on the best mapping numbers, we will perform the rest of the analysis and subsequently apply

mixOmics to understand how contaminants might be related to transcriptome and metabolome results.

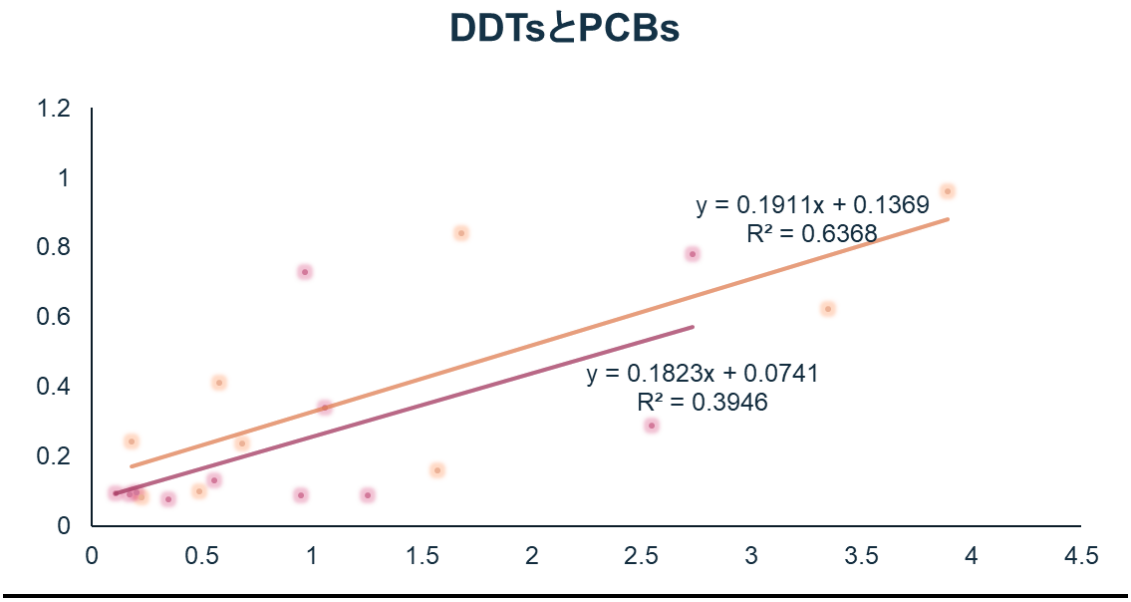

Figure 1. DDTs and PCBs linear relationships in blood plasma of *C. moreletii* from 2021.

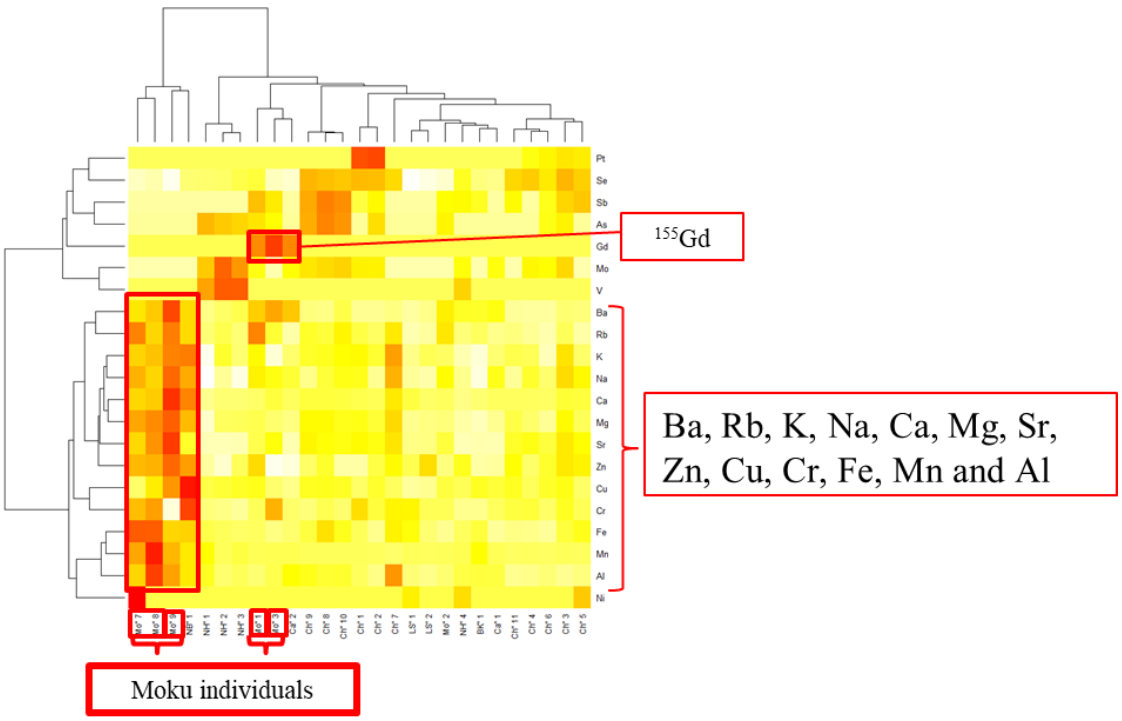

Figure 2. Heatmap analysis of trace elements in caudal scutes of *C. moreletii* collected in the Yucatan Peninsula, Mexico 2021.

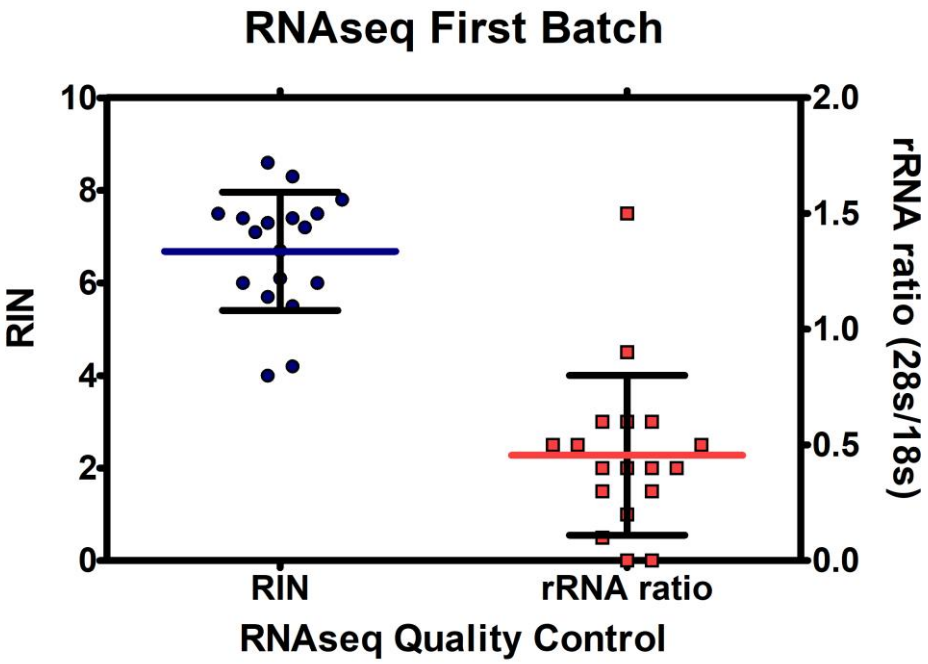

Figure 3. RNA extracts quality control for caudal scutes biopsies of *C. moreletii* collected in the Yucatan Peninsula, Mexico 2021.

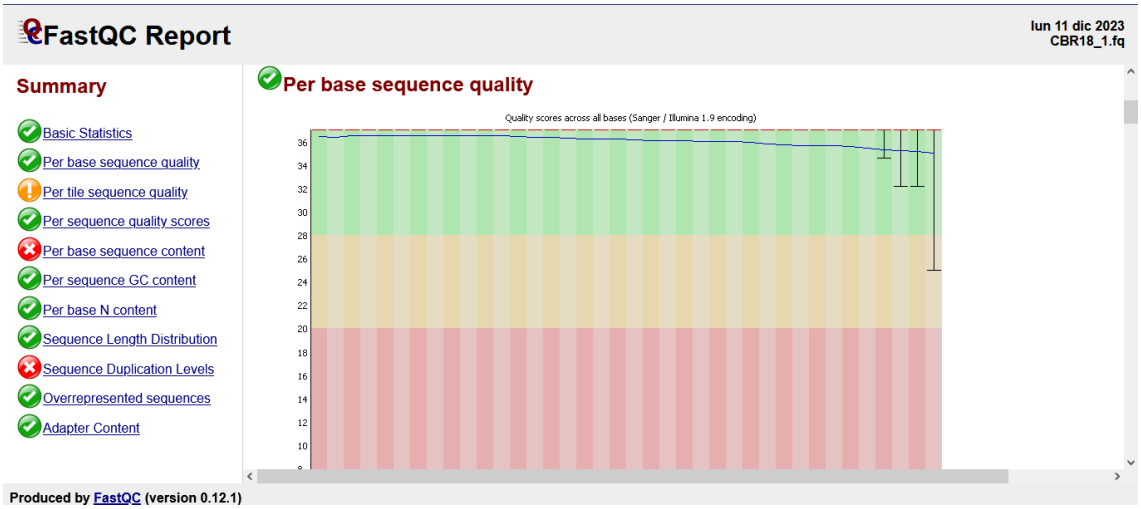

Figure 4. FastQC summary of the library generated for sample CBR18_1.

Future Challenges

1) We expected to receive 585 samples from the second batch this year. However, exportation is getting relatively problematic due to bureaucratic issues (Presidential elections). We are working our best to collaborate with the Mexican government to go through the procedure as soon as possible.

2) After receiving the second batch, we still need to consider enough budget to generate 95 libraries of RNA, based on the number of samples collected for

transcriptome.

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

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