Project Overview and Objectives

In nature, animals can be colonized by different microorganisms, or microbes, in mutualistic, commensal, and pathogenic relationships. These microbes can influence several physiological functions of their hosts (McFall-Ngai et al. 2013), such as immune response (Round and Mazmanian 2009, Hooper et al. 2012). Beyond the microbe interaction, pesticides, such as imidacloprid, can affect hosts, microbes, and parasites either directly or when associated with each other. Even being considered of moderate toxicity (in oral contact) and low toxicity (in dermal contact), unlikely carcinogen, and weakly mutagenic (EPA 2020), imidacloprid can reach the freshwater ecosystems, affecting frogs through reduction of mobility, distance, and time of swim (Sweeney et al. 2021). In this context, our proposal aims to understand how pesticides affect the immune response of Cuban tree frogs (*Osteopilus septentrionalis*), by looking at differential gene expression, exposed to symbiotic microbiomes, at natural conditions. This proposal, which is part of a larger project that aims to understand the different paths of the pesticide-microbiome-parasite interactions with each other and the immune system, labeled in Figure 1, will focus on the blood RNA samples. The RNA-seq analysis will provide information about how the pesticide treatment is impacting gene expression and the immune response.

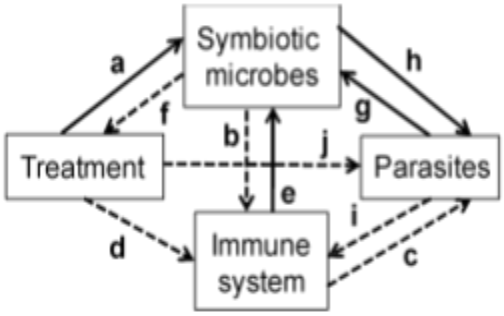

Fig. 1. Potential directional interactions (indicated with arrows) among treatment, symbiotic microbes, immune system, and parasites described in the proposed research. Solid lines indicate the pathways for which we have addressed with previous research (*paths a-h, g*) or will not be addressed (*path e*). Dotted lines indicate the pathways for which we intend on also pursuing the proposed work.

For a better understanding of the importance of the host-associated microbiota and pesticides in the development of the immune system and disease risk, Cuban tree frog tadpoles were exposed to a treatment of Imidacloprid (pesticide) or solvent control for 7 days, crossed with pond water, for the entirety of the tadpole stage. A subset of the tadpoles was sampled for their gut microbiome determination, and the rest of the individuals were kept until complete metamorphosis. Adult frogs were necropsied after three weeks and a blood sample was taken for RNA extractions. After, 8 RNA samples were sequenced using Illumina technology, with four individuals from each of the two treatment combinations (Figure 2).

Workflow

To understand how genes may be differentially expressed in the different treatment groups, we will follow a de novo RNA-seq protocol since there is not an available genome and annotation file for the Cuban tree frog. This process begins on the Xanadu cluster, with quality control and trimming of samples, followed by assembling transcriptomes. Next, coding regions

have been identified, aiming to identify and remove the clustering of redundant transcripts, and the assembly will be evaluated, annotated, and indexed. Once indexed, we generated counts of each gene, for each sample, and analyzed their expression in R software version 4.0.4 (R Core Team 2021), using the NOISeq Package (Tarazona et al. 2016).

Results and Conclusion

Trimming, quality control, De novo assembly, and transdecoder

The Samples passed in quality scores and adapter content, as shown by Final Multiqc. During the trimming process, two samples were lost, since they were almost entirely adapter content, totalizing 6 blood RNA samples. During the assembling process, a typo in one of the sample names resulted in a "prefix" file being blank, which was not included in the final assembly. Therefore, when the prefix files were concatenated, some bias may have been influenced in the results. This combined *fasta* file was then used for transdecoder, clustering, annotation, indexing, and aligning. After the identification of the open reading frames through TransDecoder, we were able to cluster similar sequences amongst the transcripts, using vsearch, resulting in a representation of every sequence to use in our centroids.fasta file.

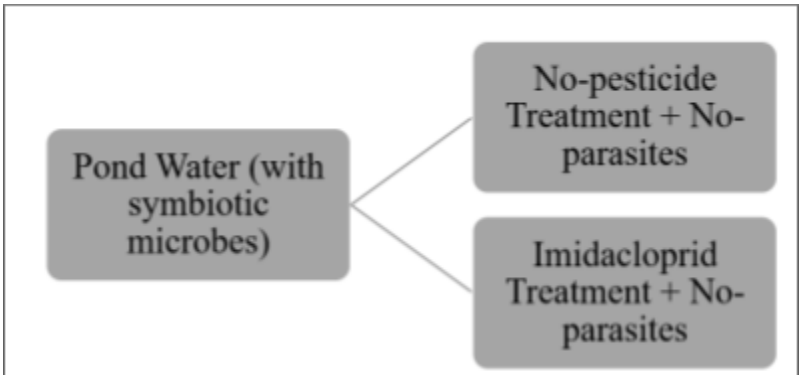

Fig. 2. Conditions for comparison of effects of a pesticide (imidacloprid), symbiotic microbes (present in pond water), and parasites in the immune system of Cuban tree frogs (*Osteopilus septentrionalis*).

RNAQuast statistical analysis and EnTAP annotation

After transcoder and clustering, we ran our transcriptome through RNAQuast, and were able to generate 42,092 transcripts, of which 16,521 had less than 500 bp and 8,357 had less than 1,000 bp. The average length of assembled transcripts was 772.23, with the longest transcript showing 20,622 of length. As a total length of sequences, we had 32,404,694, with N50 of 1,350. The EnTAP functional annotation showed minimal contamination hits, and the samples presented similar counts to other frog species, such as Western clawed frog (*Xenopus tropicalus*), with 5,100 counts, African clawed frog (*Xenopus laevis*), with 2,250 counts, and High Himalaya frog (*Nanorana parkeri*), with 4,279 counts. All the samples showed the percentage of fragments pseudoaligned equal or greater than 65% and percentage of fragments pseudoaligned to a unique target sequence equal or greater than 57%.

Gene Expression analysis

The results showed that the distribution of counts per gene was similar for all samples and treatments, which allowed us to compare the gene expression for all the final 6 blood samples. The Principal Component Analysis (PCA) showed no pattern between the treatments for gene expression. However, all samples are clustered according to the experimental design, which allowed us to proceed with the analysis for differences in gene expression. The cubic spline regression models were similar, showing that the expression of genes depends on the length, at 98.8% of dependence. Considering the two treatments, pesticide and control, at the same natural conditions (pond water), we found a total of 3,751 differentially expressed features, of which 2,755 were more expressed in control samples, and 996 were under-expressed.

In conclusion, we found that the pesticide imidacloprid influences the gene expression of Cuban tree frogs in natural conditions. As frogs were exposed to natural gut symbiotic microbes, the question "Do the host-associated microbiota mediate the effect of imidacloprid on the tree frogs?" remains. Further investigation considering the exposition to nematode parasites, as well as under no-symbiotic microbe exposure, needs to be performed, aiming to understand how imidacloprid affects the immune system of Cuban tree frogs and if the symbiotic and parasitic relationships are affected by the pesticide.

References

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