

**Title of research project:** Analysis of toxic/stress-responsive gene expression in selected Philippine wildlife species

### **Names of Project Members**

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### **Purposes**

In recent years, the escalating environmental concerns associated with persistent organic pollutants (POPs) have prompted an intensified focus on understanding their impact on diverse ecosystems. Among the affected organisms, cane toads (*Rhinella marina*) in the Philippines represent crucial subjects for investigation due to their ecological significance and potential vulnerability to environmental stressors.

Our previous study observed the accumulation of 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB153), 2,2',4,4'-Tetrabromodiphenyl ether (PBDE47) and 4,4' – Dichlorodiphenyl dichloroethylene (4'4DDE) in the tissue of organisms in Laguna Bay influencing the expression of some stress-related proteins and cytochrome P450 (CYP) enzymes in cane toads as revealed by proteomics analysis. Also, our previous study using molecular docking showed that PCB153, PBDE47, and 4,4DDE exhibited hydrophobic interactions with the binding sites of proteins. These observed direct interaction provide valuable insights into the potential disruption of expression of genes involved in xenobiotic metabolism and stress response.

Hence, this study aims to analyze the expression of *cyp1a1* and *cyp1b1* genes involved in metabolism of toxic substances, and stress-related genes in cane toads from the Philippines.

### **Methods**

#### ***RNA Isolation, cDNA Synthesis, and Quantitative Real Time-PCR***

Total RNA were isolated from the liver tissues of cane toads stored at – 80°C in CMES, Ehime University. To quantify mRNA expression levels of different genes, two step real-time RT-PCR was performed. Single-stranded cDNA from 200ug of total RNA were synthesized using High-capacity cDNA reverse transcription (RT) kit (Applied Biosystems) and ran with GeneAmp PCR System 9700 (Applied Biosystems). The cDNA were used for mRNA expression using TB Green® Premix Ex Taq II (Tli RNaseH Plus) Master Mix and analyzed using QuantStudio-3 Real-time PCR System (Applied Biosystems). The optimization of the real-time PCR reaction was performed according to the manufacturer's instructions (Applied Biosystems). The PCR conditions were 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 95 °C. The calibration curves were generated by plotting Ct values against logarithmic value of template concentration. Each reaction was run in triplicates and normalization was against the *gadh* gene.

### **Results**

#### **Xenobiotic Metabolism and Cytochrome P450s**

This study reveals varying expression levels of *cyp1a1* and *cyp1b1* in cane toads

from two sites in Laguna Bay, Philippines (Figure 1). *cyp1a1* expression is higher in Los Baños, while *cyp1b1* shows greater mRNA expression in Sta. Rosa. These cytochrome P450 enzymes serve as biomarkers for environmental pollution, responding to pollutants like PAHs, dioxins, dibenzofurans, and PCBs. The induction of these enzymes is linked to the formation of the AhR-Arnt complex in the cell nucleus (Goksøyr 1995), resulting in increased production of carcinogenic intermediates (Buters et al. 2002), and altered steroid metabolism and decreased reproductive success in fish (Arinç et al. 2000). Limited research exists on amphibian CYP1 genes beyond *cyp1a1*, emphasizing the significance of our investigation.

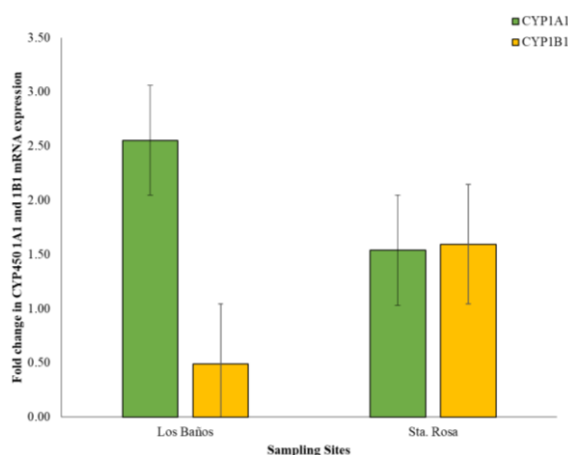


Figure 1. Hepatic mRNA expression of *cyp1a1* and *cyp1b1* from Laguna de Bay.

The use of *cyp1a1* as a biomarker is further justified by its correlation with contaminant levels in the environment. Concentrations of various contaminants in water samples and fish species in Laguna de Bay may contribute to the expression of *cyp1a1* in cane toads. This correlation extends to the induction of *cyp1b1* in both study sites. Differences in the regulation of these enzymes are attributed to the presence or absence of specific agonists, with factors like developmental stage, sex, diet, behavior, and environmental heterogeneity playing crucial roles. Additionally, the nomadic behavior of cane toads, influenced by environmental factors and body conditions (Seebacher and Alford 1999) adds complexity to the interpretation of biomarker responses in these adaptable amphibians. Nonetheless, these results help enhance our understanding of environmental pollutants' effects on crucial cellular processes, emphasizing the pivotal role of cytochrome P450 enzymes in the metabolism of toxic compounds.

## Stress Responses

### *p53*

The *p53* gene, often referred to as the "guardian of the genome," plays a crucial role in preventing the development and progression of cancer. In response to stress, beyond DNA damage, *p53* responds to hypoxia, nutrient deprivation, and oncogene activation. In these situations, *p53* activation can lead to cell cycle arrest, allowing cells to cope with stress and preventing the survival and proliferation of potentially harmful cells.

The increased expression of *p53* (Figure 2) in cane toads in Sta Rosa site reveal that these organisms are exposed to variety of stressors and may lead to modifications of the *p53* protein, such as phosphorylation and acetylation, which

in turn increase its levels and activation (Lakin and Jackson 1999).

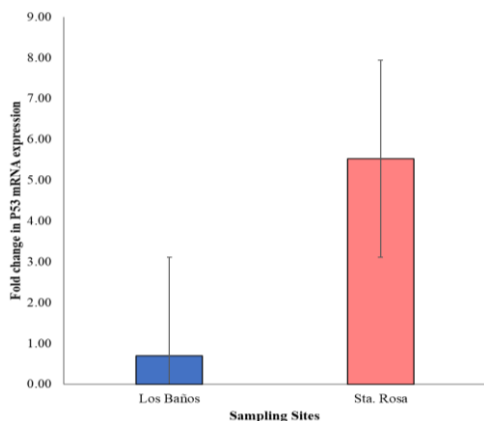


Figure 2. mRNA expression of p53 gene in *Rhinella marina*.

### Angiopoietin-like proteins (ANGPTLs) and matrix metalloproteinase-1 (MMP-1)

ANGPTLs and MMP-1 are involved in various cellular processes and understanding their roles in response to cellular stress is crucial for elucidating the complex interplay of molecular pathways involved in tissue homeostasis, repair, and adaptation to various stressors. These proteins contribute to the fine-tuning of cellular responses under stress conditions, ultimately influencing tissue function and overall organismal health.

According to Rosal (2018), the protein showing the highest upregulation in cane toads from Los Baños and Santa Rosa is *angptl7*, exhibiting fold change ratios of 40.57 and 48.52, respectively. Our findings validate this by confirming that the gene expression levels of *angptl7* are elevated in SR compared to LB ( $\Delta\text{Ct}$  SR = 9.88,  $\Delta\text{Ct}$  LB = 8.76) (Figure 3). Despite this distinction, statistical analysis reveals no significant difference between the two sites ( $p = 0.29$ ).

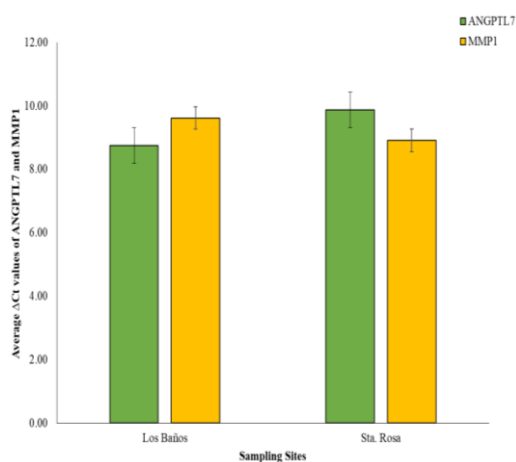


Figure 3. mRNA expression of *angptl7* and *mmp1* genes in *Rhinella marina*.

### Annexins and integrins

Annexins and integrins play important roles in stress response and provide insights into the intricate mechanisms by which cells cope with environmental challenges and maintain homeostasis. Their involvement in anti-inflammatory processes, membrane repair, and signaling makes them key players in the cellular responses to stress and injury.

Although there is no significant difference in *anxa2* and *itgb1* expression between the two sampling sites (Figure 4), these results imply comparable expression levels of the two genes among the cane toad populations in LB and SR. Under the studied circumstance, *anxa2* and *itgb1* may be genes that are stably regulated in response to the stressors or environmental factors examined.

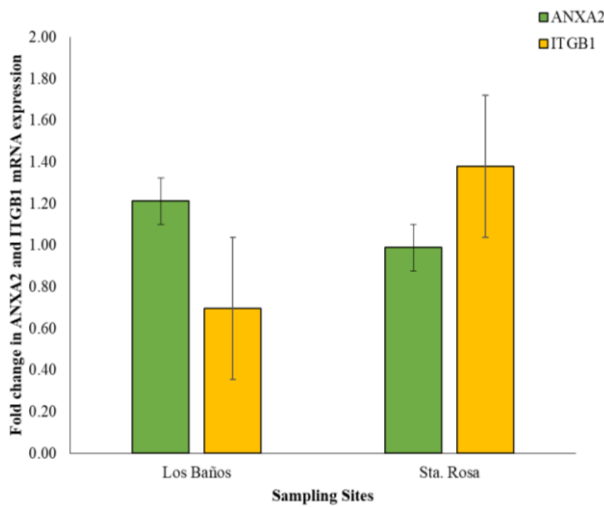


Figure 4. mRNA expression of *anxa2* and *itgb1* genes in *Rhinella marina*.

### SWI/SNF-related matrix-associated actin-dependent regulator and Protein Tyrosine Phosphatases

*smarca2*'s involvement in chromatin remodeling allows it to influence the transcription of genes involved in stress response. It may regulate the expression of genes related to cell survival, DNA repair, and other stress-related pathways. Meanwhile, *ptp4a3* is a protein tyrosine phosphatase implicated in cell migration and invasion. In stress situations, such as tissue damage or inflammation, cells may need to reposition themselves, and *ptp4a3* could play a role in these processes.

Except *smarca2* expression in Los Baños, the gene expressions decreased (Figure 5). This may suggest a reduction in the activity of the associated proteins. Genes involved in stress response often have roles in cellular adaptation and survival. A decrease in the expression of genes like *smarca2* or *ptp4a3* may indicate a compromised ability of cells to respond to stress, potentially affecting processes such as DNA repair, cell migration, or signaling.

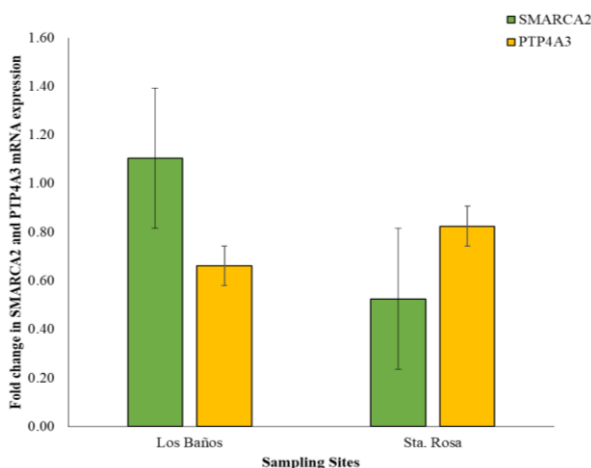


Figure 5. mRNA expression of *smarca2* and *ptp4a3* genes in *Rhinella marina*.

In summary, POPs are known to disrupt various physiological processes within organisms, including pathways associated with xenobiotic metabolism, antioxidant defense, and stress response. By unraveling the intricacies of gene expression in response to POPs, this study aims to validate and enhance our understanding of how these pollutants influence critical biological pathways in cane toads.

### **Future challenges**

The Philippines, endowed with rich biodiversity, faces the imperative to safeguard its ecosystems from the deleterious effects of environmental contaminants. Through a comprehensive examination of gene expression patterns, further research is needed to contribute essential knowledge that can inform conservation efforts, guide environmental policies, and ultimately foster the sustainable coexistence of diverse species in the face of evolving environmental challenges.

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