**The effects of 17⍺-ethinylestradiol on hepatic transcriptome of early-life stage and adult Japanese quail**

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**Introduction**

17⍺-ethinylestradiol (EE2) is an endocrine disruptor widely found in the environment. EE2 is known to cause feminization in fish[1-4] and reproductive organ abnormalities in embryonic Japanese quail (JQ)[5]. JQ has been used frequently as a research model in toxicity studies[6]. However, there is only one standardized toxicity test protocol for adult and none for early life stage (ELS) JQ. In the case of other species, researchers have used ELS under the premise that it is the most susceptible stage to toxicants[7]. Currently, there is little research on the gene expression alterations on JQ, either ELS or adult, upon the exposure to EE2. Moreover, avian RNA sequencing data has not been produced and analyzed in perspective of ecotoxicology[8] though it has huge potential to be applied for predictive toxicology[9].

**Purposes & Expected Outcomes**

In our study, we aim to determine the transcriptomic responses of ELS and adult JQ to EE2 by analyzing the first RNA sequencing data in avian ecotoxicology. According to the previous studies[5,10-11], we expect that genes such as apoVLDL, and the ones responsible for sex organ development and sexual behaviors will be detected as DEGs in both adult and ELS JQs. Furthermore, based on the comparison of ELS and adult life stages’ sensitivity to toxic chemicals in fishes and invertebrates[7], we hypothesize that comparing to adult, ELS JQ would be more susceptible to EE2 than adult, specifically ELS would have more DEGs and dysregulated pathways, and would show higher fold changes and more hits per enriched pathway as a sign for higher susceptibility to EE2.

**Experimental Design & Data Collection**

In this study, we take advantages of RNA sequencing data produced via EcoToxChip project[12]. RNAs were isolated from left-lobe livers of total 30 individuals (= 2 life stages X 3 dose groups X 5 replicates) and reverse-transcribed to cDNAs. ELS and adult JQ cDNAs were sequenced by Illumina HiSeq 4000 PE100 and Illumina NovaSeq 6000 S4 PE100, respectively. The abundance of transcripts was quantified on Galaxy using Kallisto workflow. Details of exposure experiments for embryonic and adult JQs are described below.

**ELS:** EE2 was dissolved in dimethyl sulfoxide (DMSO) to be 50 mg/mL and the dosing solution was injected into the air cell of JQ eggs at two concentrations (High: 33.3 µg/g/egg; Medium: 3.33 µg/g egg). The control group was similarly injected with dimethyl sulfoxide (DMSO). Eggs were incubated for 9 days.

**Adult stage:** Following US EPA guideline OCSPP 850.2100, Six- to ten-week old adult JQs were dosed with EE2 (High: 5 mg/kg; Medium: 0.5 mg/kg) and sampled 4 days after dosing.

**Data Analysis Approaches**

The gene expression values with the same gene names will be summed up for each sample. Then, genes with low abundance and low variance will be filtered out. Secondly, seven different normalizations including auto-scaling, trimmed mean of M-values normalization and generalized log transformation will be attempted. After confirming there is no missing value, we will examine the normalization results with box plot, density plot, histogram, heatmap, mean-standard deviation plot, some tables of statistical values (dimensions, mean, median, mode, IQR, etc), scatter plot, standard deviation plot, ‘mean - median’ plot, kurtosis plot, skewness plot and quantile-quantile plot.

Differential expression analysis will be conducted using edgeR package. Calculated parameters (logFC, logCPM, LR, p-value, and adjusted p-value) will be used to draw MA plots, volcano plots and heatmaps. Fold change and adjusted p-value will be used to select DEGs. Dysregulated pathways will be identified for each life stage using overrepresentation analysis embedded in ‘Networkanalyst.ca.’ Correlation test will be conducted on the parameters of common DEGs between ELS and adult. T-test and Mann-Whitney U-test (with multiple testing corrections) will be conducted to identify the differences between ELS and adult (both sample and feature-wise).

For unsupervised machine learnings, both (sI)PCA and clustering algorithms are applied to further investigate EE2 effects on both ELS’ and adults’ datasets. Hierarchical clustering, K-means clustering and model-based clustering with different linkages are going to be attempted.

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