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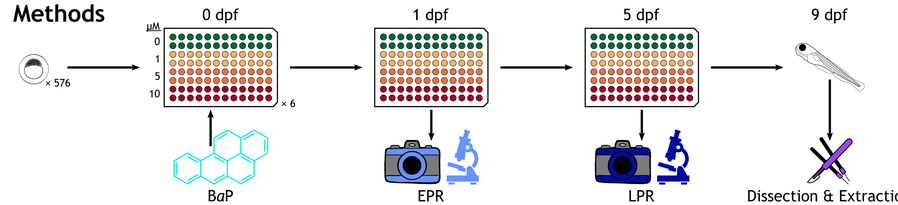
KEATON STAGAMAN, Kristin Kasshau, Robyn Tanguay, & Thomas J Sharpton

Embryonic exposure to Benzo[a]pyrene affects behavior and microbiome composition in larval zebrafish

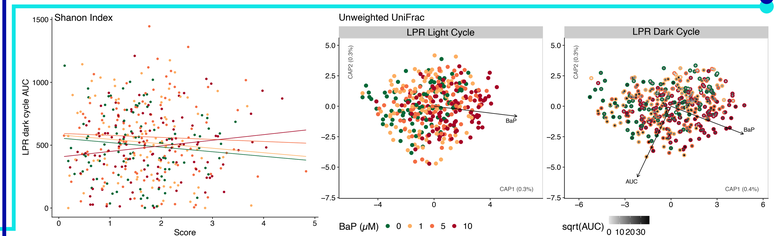
Oregon State University

Introduction. Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon, a class of ubiquitous environmental toxicants. BaP, specifically, can be found in crude oil and produced by any process that involves the incomplete combustion of organic materials, e.g. vehicle exhaust, refuse burning, cigarette smoke, and grilling foods. BaP is a procarcinogen, and the various metabolic products generated from oxidative metabolism of it exhibit a variety of reactivities, leading to complicated pathways of cytotoxicity. In zebrafish BaP has well-documented effects on neurodevelopment, resulting in specific behavioral outcomes. We hypothesized that the myriad metabolic capabilities of the intestinal microbiome contribute significantly to how BaP affects the neurodevelopment of zebrafish, with the potential for certain constituents to be protective, and other to exacerbate its effects.

Methods

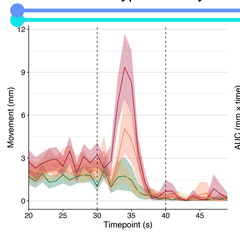


BaP influences the association between microbiota diversity/composition and larval behavior



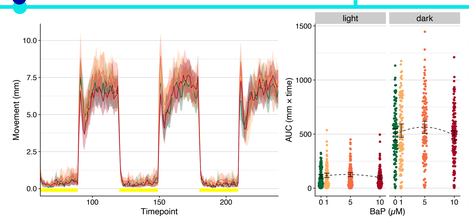
(Left) Scatter plot of LPR dark cycle AUCs by Shannon index score separated by BaP exposure level. Lines indicate linear regression estimate. (Right) dbRDA ordinations of zebrafish microbiome unweighted UniFrac metrics for LPR Light vs Dark Cycle modles. Greyscale coloring of points indicates the square-root (for visualization purposes) of the LPR AUC for the individual points. Green to red coloring of the points indicates what level of BaP exposure the individual samples were exposed to. Black arrows indicate the direction and relative magnitiude of the associations between microbiome composition and AUC and/or BaP exposure. Points are colored and arrows are only present if the association was deemed statistically significant by a PERMANOVA test.

BaP induces hyperactivity in embryonic zebrafish



(Left) Embryonic photomotor response (EPR) assay movement data. Lines show the mean movement (mm) for zebrafish embryos for each BaP exposure level (in μM). Shaded ribbons indicate 95% C.I.s for the means. Black dotted lines indicate the window of time on which later statistical analysis are based. (Right) Areas under the curve (AUCs) for the movement data, measured in the analysis window. Black error bars indicate the 95% C.I.s of the means. The black dotted line indicates the estimated association from linear regression.

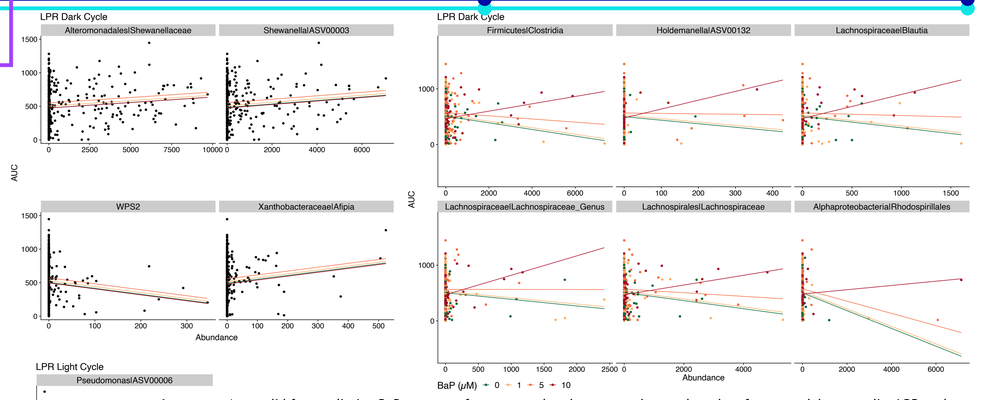
BaP induces hyperactivity* in larval zebrafish



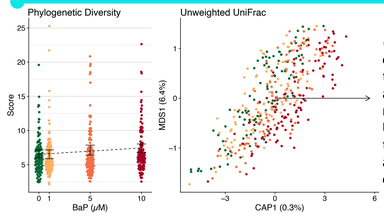
(Left) Larval photomotor response (LPR) assay movement data. Lines show the mean movement (mm) for zebrafish larvae for each BaP exposure level (in μM). Shaded ribbons indicate 95% C.I.s for the means. The yellow segments near the x-axis indicate which time points fall within light cycles (rest dark cycles). (Right) AUCs for the movement curves, split by cycle. Black error bars indicate the 95% C.I.s for means. The black dotted line indicates the estimated association from polynomial linear regression.

Certain microbes associate with behavior regardless of BaP

Other microbes associate with behavior in a BaP-dependent manner

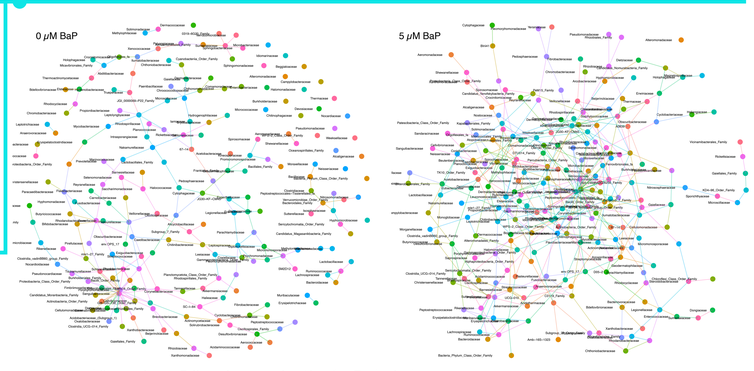


BaP induces changes in gut microbiota diversity & composition

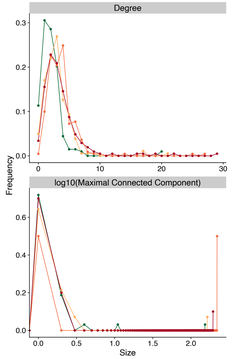


(Left) Associations between phylogenetic diversity and BaP exposure (in μM). Black error bars indicate the 95% C.I.s for the means. The black dotted line indicates the estimated association from linear regression. (Right) Distance-based redundancy analysis (dbRDA) ordination based on unweighted UniFrac scores. Points are colored by BaP exposure level and the black arrow indicates the direction of greatest change across the ordination and the relative magnitude of the effect.

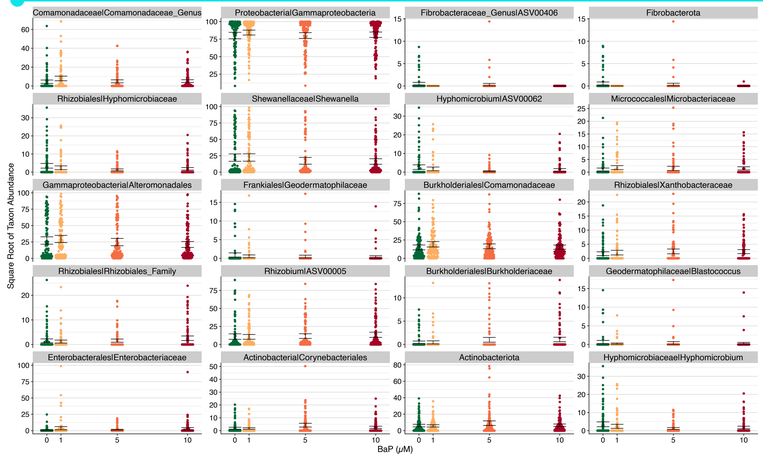
BaP induces changes in gut microbiota relational networks



We used Sparse InversE Covariance estimation for Ecological Association and Statistical Inference (SPIEC-EASI; implemented with the SpiecEasi R-package) to infer statistically significant associations between the abundances of taxa at all taxonomic levels from ASV to phylum per BaP exposure level. For illustrative purposes, we show just the associations between microbial classes for 0 and 5 μM BaP exposure levels. In each network, each node is a microbial class, and each edge denotes a significant correlation between the abundances of two classes. To the left, network metrics: (Top) distribution of node degrees [how many other nodes is each node connected to] and (Bottom) distribution of the sizes of maximal connected components [connected subgraphs of a graph to which no vertex can be added and it still be connected].



Random forest analysis identifies biomarkers of BaP exposure



We utilized two sets of rarefied taxon counts, ASVs-only and ASVs plus aggregations into taxonomic assignments, to generate both regression and classification random forest models (4 models). We split these two data sets into training and test sets (70/30), so we could assess the accuracy of the models. For whichever data set (ASV-only vs aggregated) generated the better model (see table to right), we then assessed which taxa were statistically significantly important for predicting BaP exposure. These plots show abundances by BaP exposure level for the top 20 unique most important taxa for the classification random forest model.

Taxa set	RMSE	ROC AUC
ASV-only	3.8842	0.5416
aggregated	3.9172	0.5424

As we did for predicting BaP exposure from taxon abundances we also used random forest models to predict LPR cycle AUCs. From the best random forest model for each cycle, we identified the significantly important taxa, and used mixed effects linear models to assess which taxa signficantly associated with behavior, regardless of BaP exposure level, and whether any taxa had significant interactions with BaP exposure (i.e., were there any taxa that had differing relationships with behavior depending on BaP exposure level). These plots highlight representative (not the full set) taxa that significantly predicted behavior in a BaP-independent (left panels) or BaP-dependent (right panels) manner

Conclusions and Future Directions.

BaP has modest, but significant, associations with diveristy (phylogenetic) and microbiome composition (unweighted UniFrac). One possible interpretation that is congruent with these results is that otherwise rare taxa that are capable of metabolizing BaP are increasing in abundance, though the identification of biomarkers suggests these relationships may not be linear. At lower levels of BaP exposure, we see that there is a negative or neutral assocation between microbiome diversity and hyperactivity. However, at the highest exposure level (10 μM) we see this association inverted: higher alpha-diversity associates with higher activity. Again, this may be due to some interaction between microbes that can metabolize BaP (and what those metabolic products are) and potential direct and indirect effects of these microbes on fish behavior. Utilizing random forest and linear mixed effects models, we have identified specific taxa that could have a protective effect (e.g. phylum WPS2, which as a negative association with LPR movement AUCs regardless of BaP exposure), or an exacerbative effect (e.g. various Lachnospiraceae taxa that have increasing positive associations with LPR movement AUCs with increasing BaP exposure level). Guided by these results we plan to experimentally identify protective vs exacerbative taxa (utilizing gnotobiotic zebrafish) as well as identify specific microbial metabolic functions (through metagenomic and/or metatranscriptomic) sequencing of conventiona and/or gnotobioltic microbiomes). Additionally, metabolomic studies focusing on clarifying the microbe-microbe relationships identified by the abundance network analysis here will aid in understanding how the microbiome conributes to the effects on BaP on the neurodevelopment and behavior of zebrafish, and potentially other vertebrates as well.