The Gut Microbiome Drives Benzo[a]pyrene's Impact on Zebrafish Behavioral Development

By

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Abstract

Benzo[a]pyrene (B[a]P) is a common environmental pollutant produced from the incomplete combustion of organic matter, such as fossil fuel emissions. In vertebrates, B[a]P exposure is associated with hyperactivity, though the underlying mechanisms driving this effect are undescribed. Mounting evidence indicates that the gut microbiome, which is the diverse community of microorganisms that comprise the gastrointestinal tract, could mediate the impact of B[a]P exposure on vertebrate behavior. In particular, an array of evidence raises the hypothesis that the gut microbiome's metabolism of B[a]P influences its toxicity in a way that impacts B[a]P's effect on early-life behavioral development in vertebrates. Alternatively, B[a]P could negatively alter the composition of the gut microbiome, inducing dysbiosis and impairing behavioral development. In this study, we explicitly tested these hypotheses using a zebrafish model. Zebrafish embryos colonized with conventional, conventionalized or germ-free microbiomes were exposed to varying concentrations of B[a]P and their photomotor response was assessed at 1 and 5 days post fertilization (dpf). Relative to conventionally reared zebrafish, germ-free zebrafish expressed a hyperactive photomotor response, and conventionalized expressed a hypoactive photomotor response in the dark following a light-dark transition at 5 dpf. These data suggest that presence or absence of the microbiome impacts behavioral development in juvenile zebrafish. This research builds on the body of evidence demonstrating the gut microbiome's important role influencing pollutant metabolism and impact on vertebrate neurological development.

Introduction

Benzo[a]pyrene (B[a]P) is a polycyclic aromatic hydrocarbon (PAH) formed from the incomplete combustion of organic matter and is considered by the Environmental Protection Agency (EPA) a priority environmental pollutant. B[a]P is often used as a representative model in PAH toxicology studies because it is highly characterized and correlated with other PAHs[1,2]. B[a]P can be found in the smoke of burning wood, car exhaust, factory emissions, cigarette smoke, and burnt char on food, and exposure can occur through ingestion or inhalation[1]. Once metabolized in the body, B[a]P forms a diol epoxide, which is a reactive oxygen species (ROS) that causes oxidative stress and damages cellular macromolecules including DNA, proteins, and lipids[3]. The formation of ROS causes enhanced lipid peroxidation, which lends B[a]P to be particularly neurotoxic and associated with neurodegenerative diseases[4].

Attention Deficit/Hyperactivity Disorder (ADHD) is a neurodevelopmental disease characterized by hyperactivity. It impacts approximately 6-7% of children and 2-5% of adults worldwide, and can only be managed through behavioral therapy or pharmaceuticals because no known cures exist[33,34]. Exposure to B[a]P from air pollution has been associated with prevalence of ADHD symptoms in children[2]. Additionally, B[a]P exposure adversely impacts normal neurological and behavioral development of juvenile mice and zebrafish[5–8]. In particular, Zebrafish exhibited physiological changes such as hyper photomotor activity, decreased heartbeat and mitochondrial function, and behavior deficits such as social anxiety, decreased learning and memory[7,8].

In addition to impaired neurological development, B[a]P has been shown to impact the composition of the gut microbiome, which is alarming due to the role the gut microbiome plays in vertebrate physiology[9]. The microbiome is a diverse community of microorganisms and their

metabolites associated with a host[10]. More specifically, this community can include bacteria, fungi, viruses, and archaea. Collectively, these microbes can produce a complex assemblage of metabolites that interact with their host's physiology, such as vitamins, antigens, proteins, or short chain fatty acids[10]. Increasing work demonstrates that the functioning of gut microbes can also influence neurophysiology and may impact behavioral development[11]. The observed impact of B[a]P on the gut microbiome raises concern that B[a]P may elicit toxicity by impairing the microbiome's contribution to neurophysiology. For example, disturbances to the gut microbiome during important neurological development in prenatal, early postnatal, and adolescence phases has been shown to have a negative impact on proper brain development and mental health later in life[12]. However, the mechanisms underlying how the microbiome affects neurological and physiological development remain unclear[12–14].

In this project, we investigated whether the presence or absence of the microbiome influences behavior development when exposed to B[a]P in the model vertebrate zebrafish (*Danio rerio*). Zebrafish are an ideal model vertebrate due to their similarity in early juvenile development to humans, the many well established high-throughput embryological, toxicological, and experimental methods to control the composition of their microbiomes through derivation of gnotobiotic fish, and the use of the photomotor response as a functional measure of neurological development[15–18]. We reared zebrafish with a microbiome (conventional), no microbiome (germ-free), or a microbiome that was reintroduced (conventionalized). We exposed these cohorts of zebrafish to increasing concentrations of B[a]P and measured their photomotor response at 5 days post fertilization (dpf). At 5 dpf, after performing a compound-Poisson generalized linear model on dark cycle movement data, we observed that germ-free larvae expressed a hyperactive photomotor response and conventionalized larvae exhibited a hypoactive photomotor response

compared to conventionally reared zebrafish larvae. However, after performing a linear model on light cycle data we did not observe an effect in germ-free larvae, but we did observe a hypoactive photomotor response in conventionalized larvae during the light cycle. These findings indicate that the presence or absence of the microbiome differentially impacts behavioral development in juvenile zebrafish.

Results

Observed associations between embryo photomotor response (EPR), B[a]P concentration and microbiome treatment at 1 day post fertilization (dpf)

To determine if the presence or absence of the microbiome impacts behavioral development in zebrafish exposed to B[a]P early in life, we measured embryo and larvae photomotor response at 1 and 5 days post fertilization (dpf). Photomotor response is a measure of movement in reaction to a light stimulus, and can be used as a functional measure for neurological development in zebrafish. We used the measurements obtained from the photomotor response to calculate the mean total movement over time per treatment group and B[a]P concentration. The total movement was then used to statistically analyze the area under the curve (AUC) for each treatment and compare activity levels to assess behavioral development (Fig. 1).

At 1 dpf, we compared germ-free and conventionalized embryo photomotor response (EPR) to conventionally reared embryos. AUC's for individual embryos were not normally distributed and the data fit best to a compound-Poisson generalized linear model (CPGLM) (Fig. 2). We did not observe a concentration-dependent EPR in either the germ-free (GF) or conventionalized (CVZ) microbiome treatments relative to conventionally (CV) reared embryos (Table 1). However, a

CPGLM was performed to predict the movement by combining microbiome treatment groups of all embryos. We observed a slight hypoactive negative association between embryo movement and B[a]P concentration ($t(1388) = -1.977$, $p = 0.0482$, Fig. 2 & Table 1). Embryos predicted movement was equal to 31.584 - 0.904 (B[a]P Conc) where movement was measured in millimeters (mm) and B[a]P was measured in micromolars. Embryo movement decreased by 0.904 for every micromolar of B[a]P. Additionally, because the data fit a nonparametric distribution, we conducted a ranked based analysis of variance (ANOVA) to compare the effects of B[a]P concentration and microbiome treatment on movement. We observed a statistically significant relationship between AUC ranks for individual embryo movement and at least one microbiome treatment group ($F = 5.42$, $p = 0.005$, Table 2; Fig. 3). It is likely that this difference was driven by the difference we observed between germ-free relative to the conventional treatment as seen in the Poisson model ($t(1388) = -1.433$, $p = 0.1521$; Table 1). Thus, at 1 dpf our results suggest it is too early to detect a microbiome effect on movement and B[a]P exposure in embryos.

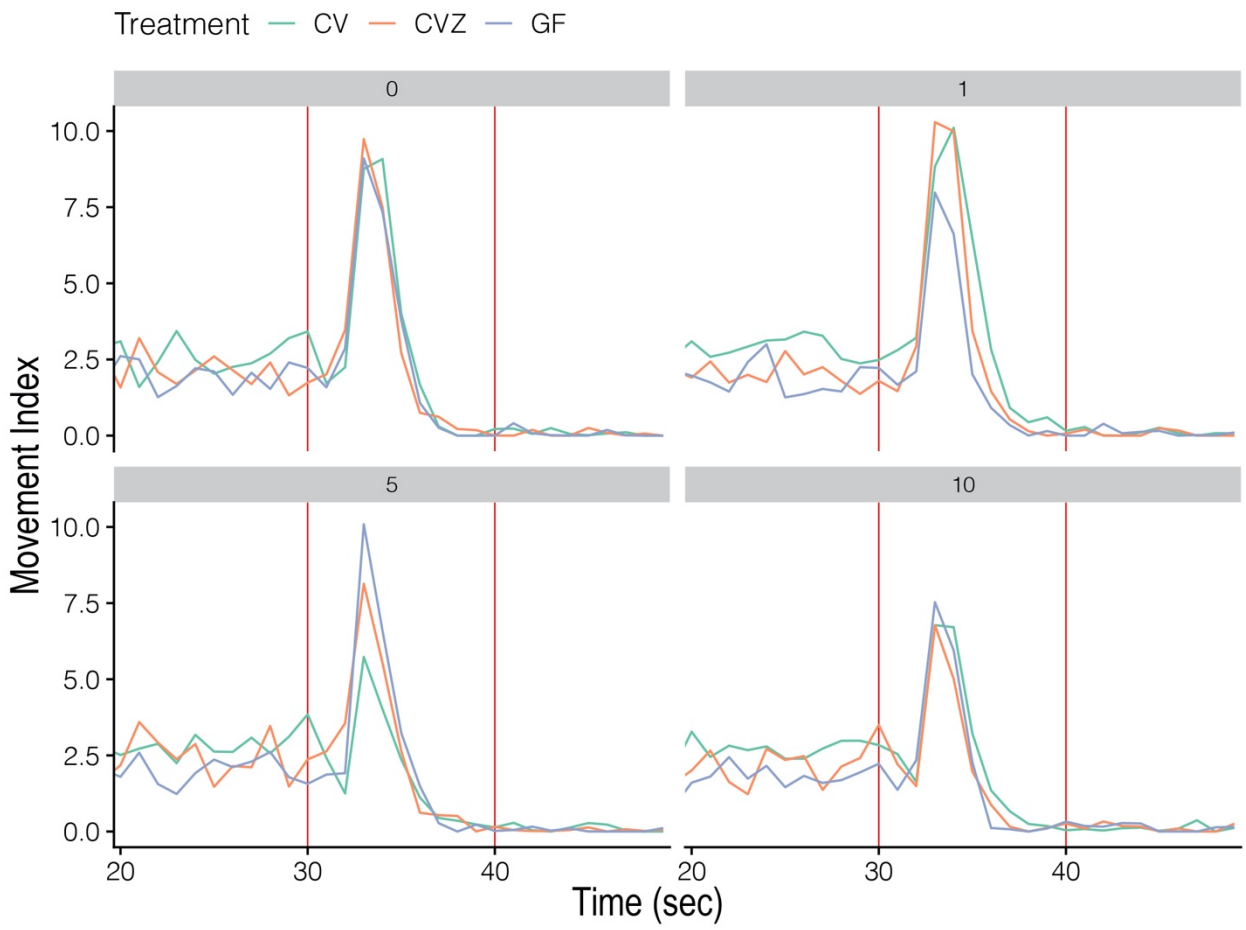

Figure 1: Mean movement of area under the curve (AUC) by microbiome treatment and B[a]P concentration for EPR analysis at 1 dpf. Plot of movement over time. Lines indicate mean movement per microbiome treatment. Vertical red lines indicate the window of movement we analyzed statistically. Subplots broken up by B[a]P concentration (0, 1, 5, and 10 μM respectively).

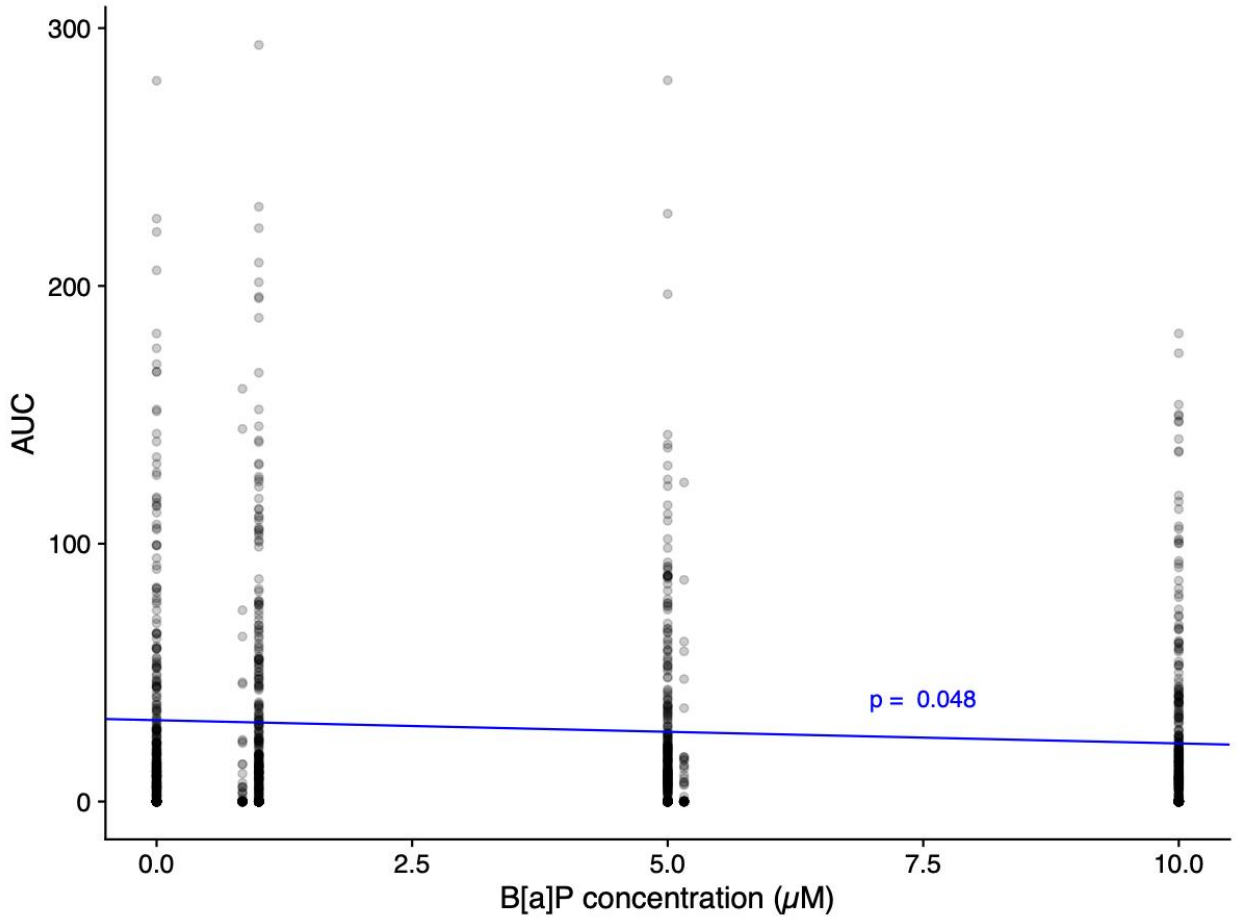

Figure 2: Area under the curve for movement by B[a]P concentration at 1 dpf. A compound Poisson generalized linear model was fit to the data using B[a]P concentration in micromolars and area under the curve (AUC) for movement. Dots represent individual embryos. n = 343 (0μM), 348 (1μM), 351(5μM) and 352 (10μM).

Table 1: Summary statistics for compound Poisson generalized linear model in Figure 2.

CPGLM	Estimate	Std.Error	t-value	Pr(>|t|)	
(Intercept)	31.58	2.92	10.83	<2e-16	***
B[a]P Conc	-0.9	0.46	-1.98	0.048	*
TreatmentCVZ	-1.51	4.09	-0.37	0.713	
TreatmentGF	-5.54	3.86	-1.43	0.152	
B[a]P Conc:TreatmentCVZ	-0.04	0.63	-0.06	0.95	
B[a]P Conc:TreatmentGF	0.49	0.62	0.79	0.431	

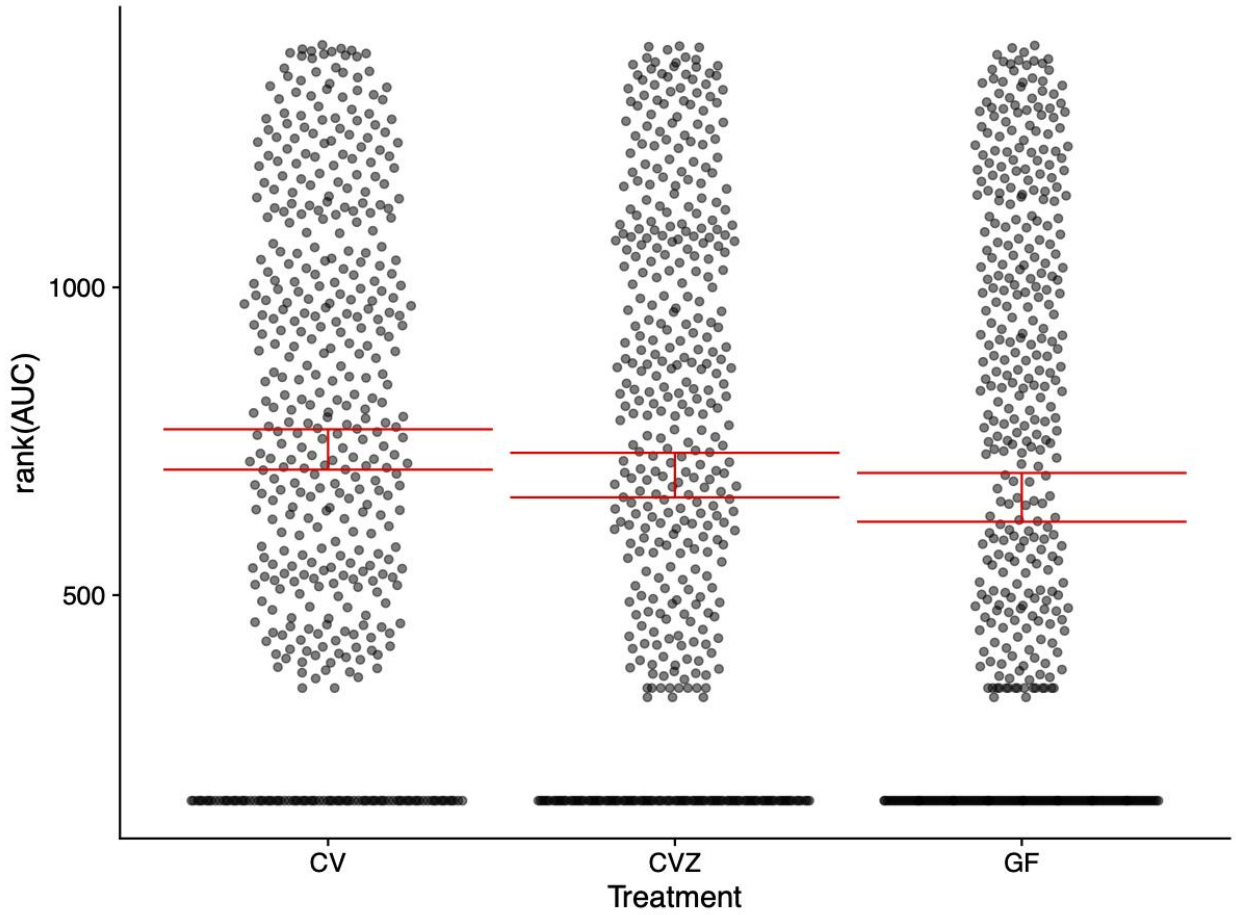

Figure 3: Area under the curve ranks for individual embryo movement by microbiome treatment at 1 dpf. Ranks of AUC data by microbiome treatment. Red bars indicate bootstrapped 95% confidence interval. n = 470 conventional (CV), 467 germ-free (GF), and 457 conventionalized (CVZ).

Table 2: Summary statistics for ranked ANOVA in Figure 3.

ANOVA of Aligned Rank Transformed Data	Df	Df.res	F-value	Pr(>F)	
B[a]P Conc	3	1382	1.74	0.157	
Treatment	2	1382	5.42	0.005	**
B[a]P Conc:Treatment	6	1382	1.62	0.139	

B[a]P concentration-dependent larval photomotor responses dependent on microbiome treatments at 5 dpf.

To determine if behavioral development at 5 dpf is influenced by presence or absence of microbiome, we conducted a compound-Poisson generalized linear model (CPGLM) and a linear model to predict larval photomotor responses (LPR) of larvae based on their microbiome treatment of either germ-free or conventionalized relative to conventional, following light-dark transitions in light and dark cycles respectively. Before comparing the effects of B[a]P concentration and microbiome treatment, we calculated movement based on microbiome treatment alone. In the light cycle, a significantly different LPR of conventionalized larvae relative to conventionally reared larvae was not found ($t(1254) = 1.136$, $p = 0.256$; Table 4), but we did observe a hyperactive LPR in germ-free larvae ($t(1254) = 2.723$, $p = 0.00656$; Table 3). Germ-free larvae's predicted movement was equal to 9.844 millimeters (mm) when B[a]P was not present. In the dark cycle, using a linear model we saw a stronger hyperactive relationship between LPR and germ-free larvae relative to conventional larvae ($F(5, 1254) = 10.64$, $p =$ 1.54e-05; Table 3). Germ-free larvae's movement was equal to 36.612 mm when B[a]P was not present.

To identify whether there was a combined effect on larval movement between B[a]P concentration and presence of the microbiome, we constructed an additional model that included B[a]P concentration as an additional covariate. In the light cycle, conventionalized zebrafish larvae had a significantly concentration-dependent hypoactive LPR relative to conventional larvae ($t(1254) = -2.217$, $p = 0.027$, Table 3; Fig. 5a). Conventionalized larvae's predicted movement was equal to 37.310 + 9.844 (TreatmentCVZ) - 1.369 (B[a]P Conc), where movement was measured in mm and B[a]P in micromolars. Conventionalized larvae's movement decreased 1.369 for every micromolar of B[a]P. A significant effect was not found in germ-free larvae. In

the dark cycles, for both germ-free and conventionalized larvae a significant concentration-dependent LPR was observed relative to conventional ($F(5, 1254) = 10.64$, $p = 0.0243$ & $p = 0.0158$, Table 4; Fig. 5b). Germ-free larvae's predicted movement was equal to 145.108 + 35.612 (TreatmentGF) - 3.293 (B[a]P Conc). Germ-free larvae's movement decreased 3.293 mm for every micromolar of B[a]P. Conventionalized larvae's movement was equal to 145.108 - 2.820 (TreatmentCVZ) - 3.738 (B[a]P Conc). Conventionalized larvae's movement decreased 3.738 mm for every micromolar of B[a]P. Thus, zebrafish behavior during dark cycles was dependent on presence or absence of microbiome and concentration of B[a]P. Additionally, germ-free and conventionalized larvae have a negative concentration-dependent association with activity level.

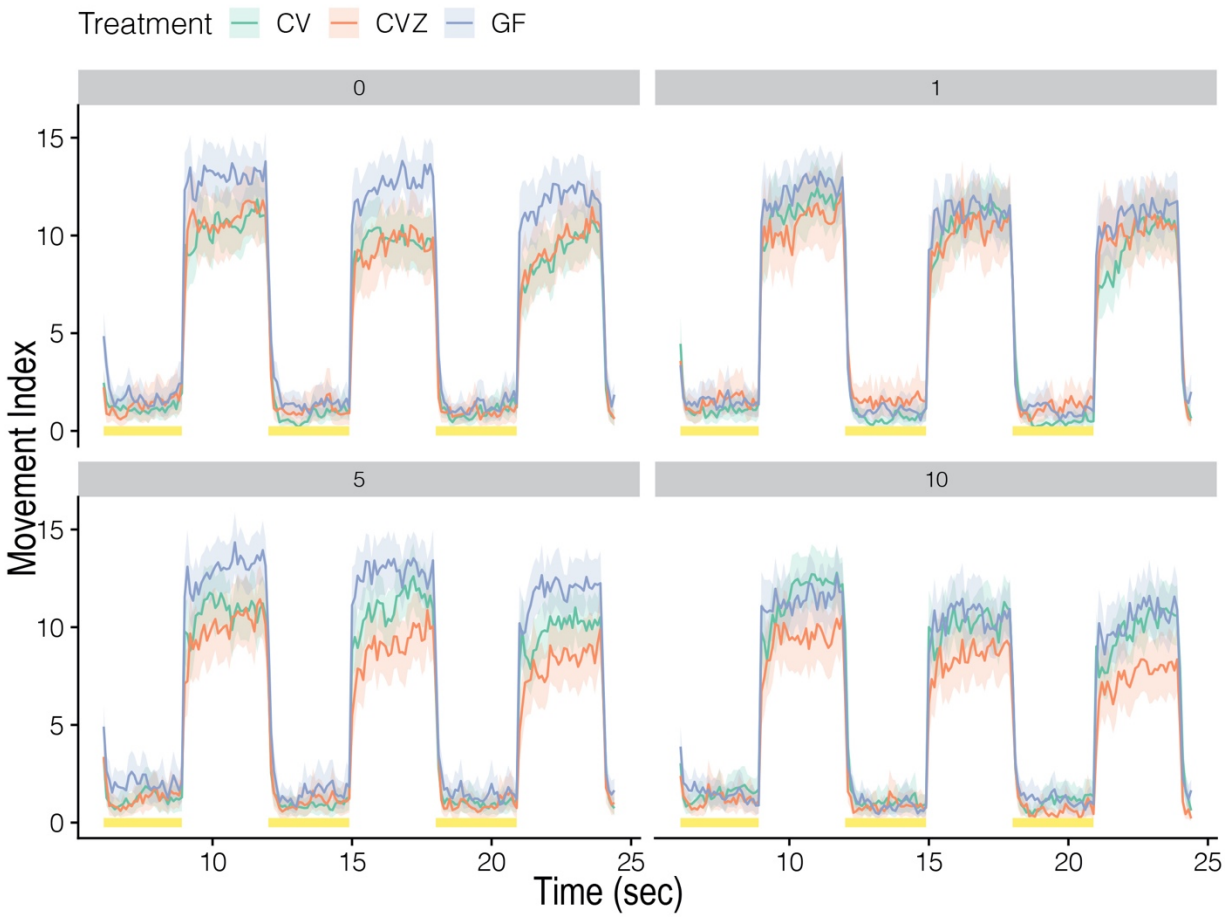

Figure 4: Mean movement of area under the curve (AUC) by microbiome treatment and B[a]P concentration for LPR analysis at 5 dpf. Plot of movement over time. Lines indicate mean movement per microbiome treatment. Horizontal yellow bars indicate light phase. Shading of lines indicates bootstrapped 95% confidence interval. Subplots broken up by B[a]P concentration (0, 1, 5, and 10 μM respectively).

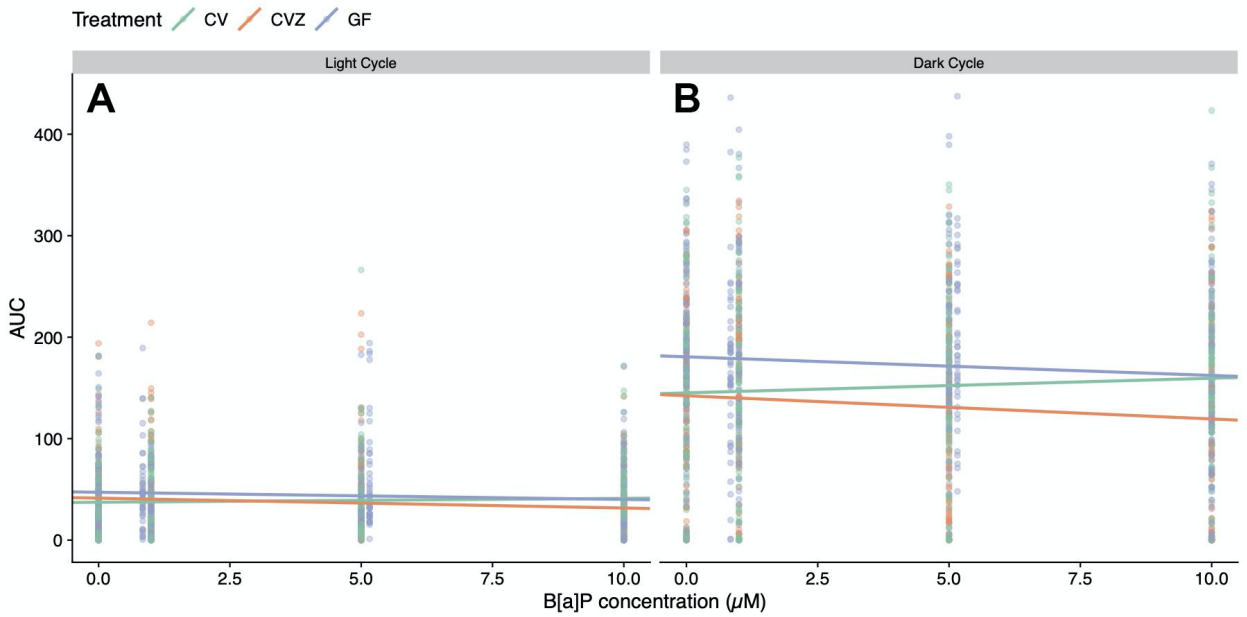

Figure 5: Area under the curve for movement by B[a]P concentration and microbiome treatment at 5 dpf. (**A**) A compound-Poisson generalized linear model was fit to the data using B[a]P concentration in micromolars and area under the curve (AUC) for movement for the light phase (left) and (**B**) a linear model fitted to the data using B[a]P concentration in micromolars and area under the curve (AUC) for movement for the dark phase (right). Dots represent individual embryos colored by microbiome treatment (green = conventional (CV), orange = conventionalized (CVZ), and blue = germ-free (GF)). n = 343 (0 μM), 348 (1 μM), 351 (5 μM) and 352 (10 μM).

Table 3: Summary statistics for compound Poisson generalized linear model in Figure 5a.

CPGLM	Estimate	Std.Error	t-value	Pr(>|t|)	
(Intercept)	37.31	2.32	16.09	<2e-16	***
B[a]P Conc	0.39	0.43	0.9	0.37	
TreatmentCVZ	4.16	3.66	1.14	0.256	
TreatmentGF	9.84	3.62	2.723	0.007	**
B[a]P Conc:TreatmentCVZ	-1.37	0.62	-2.217	0.027	*
B[a]P Conc:TreatmentGF	-1.09	0.63	-1.731	0.084	

Table 4: Summary statistics for linear model in Figure 5b.

LM	Estimate	Std.Error	t-value	Pr(>|t|)	
(Intercept)	145.11	5.77	25.17	<2e-16	***
B[a]P Conc	1.45	1.03	1.41	0.16	
TreatmentCVZ	-2.82	8.81	-0.32	0.749	
TreatmentGF	35.61	8.21	4.34	1.54E-05	***
B[a]P Conc:TreatmentCVZ	-3.74	1.55	-2.42	0.016	*
B[a]P Conc:TreatmentGF	-3.29	1.46	-2.26	0.024	*

Discussion

Benzo[a]pyrene is a common environmental pollutant associated with early-life behavioral disorders, such as ADHD, as well as dysbiosis of the gut microbiome in humans[1,2,9]. The gut microbiome is known to metabolize pollutants, and alterations of the gut microbiome composition has been associated with behavioral disorders[14,19]. However, the relationship between the gut microbiome's metabolism of pollutants and the effect that it has on behavioral development remains unclear. The purpose of this study was to elucidate the gut microbiome's influence on B[a]P toxicity and its subsequent impact on early-life behavioral development. Currently, no known cures exist for ADHD, and if a microbiome effect is observed between B[a]P toxicity and behavioral development, it could provide opportunities for novel microbiome targeted therapies.

To investigate the microbiome's role on B[a]P's toxicity and its influence on behavioral development we used zebrafish as a model vertebrate organism, exposing 1,394 embryos to varying concentrations of B[a]P, and measured their photomotor response at 1 and 5 days post fertilization (dpf). In line with previous research studying the effects of B[a]P on behavioral development[7,8], we observed that juvenile zebrafish with conventionally colonized gut microbiomes expressed a positive trend between B[a]P concentration and hyperactive larval photomotor response (LPR) in the dark following light-dark transitions at 5 dpf. Interestingly, we observed a negative trend between LPR and B[a]P concentration in zebrafish larvae colonized with germ-free and conventionalized gut microbiomes at 5 dpf. Germ-free larvae expressed a hyperactive LPR relative to conventional larvae at 0 μM of B[a]P, but their activity decreased with increased concentrations of B[a]P. These results are consistent with studies demonstrating that a lack of a microbiome is associated with hyperactivity in juvenile zebrafish[18,20], as well as

studies in germ-free mice showing differences in behavioral development compared to conventionally reared mice[21–23]. Additionally, we have built upon these studies by developing novel high-throughput methods to generate robust sample sizes of germ-free zebrafish embryos to better understand how the microbiome influences host behavioral and physiological development.

In larvae colonized with conventionalized microbiomes, we did not observe a difference in activity during the dark phase compared to conventional larvae exposed to 0 μM of B[a]P. However, as B[a]P concentration increases, conventionalized larvae became increasingly hypoactive relative to conventional larvae. This divergence in activity level as B[a]P concentration increases could have two possible explanations. First, the differences in microbial metabolism of B[a]P affecting its toxicity and its subsequent impact on neurological development. The difference in B[a]P metabolism could be attributed to different microbial communities between these two groups. The differing compositions could originate from the germ-free derivation, which removes microbes that strongly adhere to the chorions of conventionalized embryos. Conventionalized embryos would therefore lack any maternal microbes found in the spawning tanks that conventional embryos might have attached to their chorions. Wiles *et al.* showed that certain microbes exhibit competitive exclusion or a priority effect during initial larval gut colonization[24]. These maternal microbes could be responsible for the difference in microbial communities between these two groups. If B[a]P metabolism differs between the gut microbiota of conventional and conventionalized larvae, this could explain the opposing trend we observed in their LPR results. Second, the difference in activity levels could alternatively be explained by B[a]P perturbing the gut microbes, inducing dysbiosis and consequently impairing behavioral development of the larvae. Carlson *et al.* found that infant gut

microbiomes were strongly associated with cognitive development and in line with studies in mice exhibiting anxiety-like symptoms following gut microbiome disturbance[21-23,32]. Further investigation should be conducted comparing the microbial composition of embryo media, gut microbiomes of conventional and conventionalized larvae, and how these potentially different microbial communities are metabolizing B[a]P in order to better understand these observed differences.

Previous research has shown that zebrafish express a positive association between hyperactivity and B[a]P concentration at 5 dpf, but to our knowledge this link has not been measured at 1 dpf [7,8]. To determine if there was an earlier B[a]P effect on behavioral development, we measured embryo photomotor response (EPR) at 1 dpf. We observed a slight negative association between movement and B[a]P concentration. This contradicts previous research in juvenile zebrafish showing B[a]P expressing hyperactivity at 5 dpf[7,8]. There could be several reasons for the discrepancy between our and those two studies. Firstly, B[a]P may not manifest toxicity this early in development. Secondly, because chorions of the zebrafish embryos are still intact at 1 dpf and they typically do not hatch until 2 or 3 dpf, this would restrain their movement more than larvae whose chorions have hatched by 5 dpf. Thirdly, to maintain sterility we affixed transparent silicon seals to the top of the 96-well plates containing the zebrafish embryos, whereas those previous studies did not use seals. Refraction caused by light passing through the material of the seal or the accumulation of moisture on the underside of the seal could have lowered image fidelity. Together these reasons could account for the differences in movement trends we observed at 1 dpf between our study and the previous two studies.

Additionally, at 1 dpf, we did not observe a relationship between microbiome treatments when compared to B[a]P concentration and movement. This could also be attributed to B[a]P not yet

manifesting toxicity in this stage of development. Furthermore, chorions of zebrafish embryos do not hatch and their mouths do not develop until 2 or 3 days post fertilization[25]. The chorions act as a protective barrier between the embryo and its environment[25]. Microbes would be too large to pass through the chorion's membrane and would not be able to enter embryo guts until after their mouths develop at 3 dpf. We did observe microbiome treatment-dependent differences in activity level at 1 dpf, but they were not significantly different from one another. These data suggest that a B[a]P concentration-dependent relationship between microbiome treatment and EPR may not manifest at 1 dpf.

An important limitation to this study was that we did not perform 16S rRNA analysis on the conventional and conventionalized gut microbiomes of larval zebrafish. Therefore, we are unable to confidently determine whether the behavioral effect differences we observed between conventional and conventionalized larvae can be attributed to specific microbial compositions. Future research including 16S rRNA will be needed to confirm that microbial compositional differences impact B[a]P metabolism and behavioral development. Additionally, we did not culture microbes found in larval guts, which could help us better understand how microbes are metabolizing B[a]P and contributing to its toxicity. Finally, our study investigated the effects on juvenile zebrafish, but previous research by Knecht *et al.* found that behavioral disorders caused by early-life B[a]P exposure persisted into adulthood. Extending our study into adulthood could provide more insight into the long-term behavioral effects of early-life gut microbiome disruption.

Taken together, our results demonstrate that the microbiome influences the effects of B[a]P metabolism and impacts behavioral development in juvenile zebrafish. While the exact mechanisms that the microbiome uses to drive B[a]P's influence on behavioral development

remains unclear, our study builds on the body of evidence implicating the gut microbiome's central role in behavioral development in vertebrate mammals. Further investigation is needed to elucidate these mechanisms, to determine which microbes protect or exacerbate behavioral development, and what effects persist into adulthood.

Materials and Methods

Zebrafish husbandry

All experiments were conducted at the Oregon State University Sinnhuber Aquatic Research Laboratory (SARL) using wildtype 5D Tropical zebrafish (*Danio rerio*). Adult zebrafish were maintained in a recirculating system with a 14h light: 10h dark photoperiod with a water temperature of 28+/- 1°C. All embryos for exposure experiments were collected following group breeding of adult zebrafish as described previously[26,27]. Fish husbandry, reproductive techniques, exposure experiments and adult learning tests were conducted according to Institutional Animal Care and Use Committee protocols at Oregon State University.

Chemicals

Benzo[a]pyrene (B[a]P) was obtained from Sigma Aldrich and stock solutions were prepared as previously described[8]. Briefly, stock solutions were made to a stock concentration of 20 mM had 100% DMSO. Intermediate B[a]P dilution standards of 1, 5, and 10 μL were prepared fresh daily from stocks in embryo medium (EM)[25].

Germ-free derivation

For the germ-free and conventionalized treatments, we followed the germ-free derivation protocol as previously described[16]. Briefly, following spawning, Zebrafish embryos are suspended in embryo media (EM) containing antibiotics (Ampicillin (100 μg/mL), Amphotericin

B (250 ng/mL), Gentamycin (10 μg/mL), Tetracycline (1 μg/mL), Chloramphenicol (1 μg/mL)) for four hours, then washed in 0.1% poly(vinylpyrrolidone)-iodine (PVP-I) solution for two minutes, washed in 0.003% bleach solution for 10 minutes. All solutions were filter sterilized. To control for the germ-free derivation process, a subset of Germ-free (GF) embryos were removed from sterile conditions and conventionalized by placing them into polystyrene 96-well plates containing 100μL EM inoculated with microbes and fecal matter derived from tanks of conventionally reared adult zebrafish[20,28]. GF embryos were placed in polystyrene 96-well plates containing 100μL filter sterilized EM and sealed with PCR film under sterile conditions.

Benzo[a]pyrene exposures

Benzo[a]pyrene (B[a]P) exposures were performed as previously described[8]. In brief, zebrafish were statically exposed to 1, 5, or 10 μM B[a]P starting at 6 hours post fertilization (hpf) to 5 dpf in 96-well polystyrene plates, with one embryo per well in 100 μM of solution. These concentrations were selected based on previous zebrafish studies and zebrafish assays[8].

Behavioral and developmental assays

Behavioral and developmental assays were performed as previously described[17,29]. In brief, at 24 hours post fertilization (hpf), we conducted an embryonic photomotor response (EPR) assay and evaluated for mortality and 4 morphological endpoints. The EPR is a custom-built instrument called High-resolution Motion and Analysis Tracking (HMAT)[29]. The photomotor response is used as a functional measure of neurological development in zebrafish. The assay is a total of 50 seconds, with a light cycle that consists of two pulses of light. The first 30 seconds before the first pulse captures the background, with the 10 seconds following called the excitatory phase, then a second light pulse. Following this pulse of light, for 10 seconds, the phase is called refractory. A total of 800 images were acquired in this time and processed using a custom motion

detection algorithm and analyzed using custom R scripts[30]. At 5 dpf, we conducted a larval photomotor response (LPR) using the Viewpoint Zebrabox systems (Viewpoint Behavior Technology) to measure photo-induced larval photomotor activity and subsequently impute behavioral changes in swimming patterns and total movement. The assay consists of 4 cycles of 3 minutes of lights on and off. Following the behavioral assay, we evaluated mortality and 18 physiological malformations using a dissecting scope[8]. All assays were conducted between 11:30am and 4pm.

Sterility Checks

Sterility was assessed following germ-free derivation and at 5 days post fertilization (dpf) following the larval photomotor response (LPR) assay. Following germ-free derivation, 1 mL of media from the petri dish containing the germ-free embryos was pipetted onto a tryptic soy agar (TSA) plate. Following the 5 dpf LPR assay, 10 μL EM from 9 wells containing GF zebrafish was pipetted onto a (TSA) plate. Plates were incubated under aerobic conditions at 26°C for at least 7 days. Contaminated 96-well plates were excluded from the study.

Behavioral statistics

Statistical analysis of embryonic photomotor response (EPR) and larval photomotor response (LPR) assays were performed as previously described[8,29]. Briefly, raw data collected from HMAT and Viewpoint Zebrabox systems were processed using custom R (R Core Team 2020) scripts, and used to track total distance traveled and compute area under the curve (AUC) for EPR and LPR behavioral assays respectively[31]. Any mortalities or animals with physical malformations were excluded from the data analysis.

For 1 day post fertilization (dpf) statistics, we fit data using a compound-Poisson generalized linear model to plot AUC for individual wells (larvae) by benzo[a]pyrene concentration and rank-based (non-parametric) analysis of variance to plot AUC ranks for individual wells by microbiome treatment to determine if treatments are statistically different from the estimated intercept for the conventional (control) group.

For 5 dpf statistics, we fit data using compound-Poisson generalized linear model to plot AUC for individual wells (larvae) by benzo[a]pyrene concentration for the light cycle and a linear model for the dark cycle to determine if treatments are statistically different from the estimated intercept for the conventional (control) group.

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