Bioenergetic and Fitness Costs of PAH Adapted *Fundulus heteroclitus* to Early Life PAH and Hypoxia Exposures

by

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Executive Summary

Polycyclic aromatic hydrocarbon toxicity (PAH) to aquatic life has become a major area of concern in the field of ecotoxicology due to the propensity of PAHs to absorb across cellular membranes, the potency of PAH mitochondrial toxicity at low concentrations, and a history of considerable aquatic coal pollution without appropriate remediation. Despite the growing body of published research describing the mechanisms of PAH toxicity and its targeted effects on the mitochondria, very little work has been done that investigates the potential behavioral and bioenergetic costs associated with PAH adaptation – a phenomenon that has occurred in a subpopulation of killifish living in polluted waters. In addition to this, virtually no studies have done this incorporating multiple stressors that are likely to co-occur with PAH exposure, such as depleted dissolved oxygen (hypoxia).

The Duke University Superfund Research Center has long investigated the impacts of creosote discharge in the Elizabeth River, VA after many decades of site contamination from wood treatment facilities. Creosote, being a complex mixture of PAHs, binds to the sediments of the Elizabeth River where it remains for years. Remobilization of fine sediments provides an exposure route for fish endemic to these highly polluted sites, and fish living in these areas are exposed quite early in development. The lasting impacts of these early exposures are unknown. Interestingly, *Fundulus heteroclitus*, or Atlantic killifish have also been shown to have embryotoxicity resistance from PAHs but with a suspected fitness tradeoff and reduced tolerance to other environmental stressors.

The basis of my study, and my first objective, uses Atlantic killifish as a model to determine if early life acute exposures to PAHs or hypoxia may lead to later life fitness consequences. My experimental design has fish embryos dosed once with environmentally relevant PAH mixtures and hypoxia with subsequent development in clean laboratory conditions. After six months of development they undergo swimming tests to determine if locomotor capabilities have been significantly altered.

Nested in this objective is an examination if early life co-exposure to PAH mixtures and hypoxia lead to elevated toxicity relative to just a single exposure. A PAH and hypoxia co-exposure is both environmentally and mechanistically relevant. The toxicity-mediated signaling pathway for both exposures share a common protein which promotes molecular crosstalk in the event of co-exposure. It stands to reason that a co-exposed group may respond differently.

The third objective in my study then provides insight if a population of killifish adapted to PAH pollution respond differently – in terms of sustained swim performance – than those that have not adapted. Killifish that have adapted protective mechanisms for PAH toxicity would not be expected to have their locomotive capabilities significantly affected by early life exposures.

Finally, the fourth objective investigates if a decline in swimming abilities can be explained by altered mitochondrial function. To do this study, heart and brain tissue from juvenile killifish were dissected and used in a biological assay to measure oxygen consumption rate.

This last study uses well-documented chemicals that target the mitochondria in order to induce maximal oxygen consumption and to completely inhibit aerobic respiration. By doing this, my study was able to determine whether oxygen consumption by mitochondria alone is significantly different at rest or at maximum activity.

When just comparing sustained swim performance, adapted killifish do not respond differently to PAH or hypoxia exposure compared to controls. Despite no significant loss in fitness, mitochondrial oxygen consumption in heart tissue has a marginally significant increase for hypoxia exposed fish ($p = 0.07$) – an indication that organismal adaptation may lead to altered mitochondrial physiology. Non-adapted fish have significant decreases in swim performance with developmental PAH and hypoxia exposure, which is partially reflected by altered mitochondrial physiology with PAH exposure. These alterations are somewhat paradoxical as oxygen consumption appears to increase with exposure. Because of this, mitochondrial oxygen consumption alone cannot fully explain why fish adapted to PAHs have reduced locomotor stamina. This study both benefits and is somewhat complicated by many parameters – in fact, secondary environmental stressors are often unincorporated into ecotoxicology experimental design. However, the results give indication that developmental exposure, the type of exposure, and the population history may all play a role in how animals within the same species differ physiologically in response to environmental contaminants.

This novel study provides several new insights into the lasting fitness and bioenergetic alterations in killifish due to PAH adaptation and early life-stage exposures. In summary:

- Previous experimental studies have shown that killifish populations born in the Elizabeth River, VA have developed innate resistance to PAH toxicity due to high sediment loads. These high sediment concentrations were essentially created by a long history of industrial pollution.
- Multiple linear regression models indicate that a killifish population that has had historically little PAH exposure has a higher sustained swimming performance than killifish that became adapted to PAHs.
- Killifish population origin may play a large role in determining how individuals respond to PAHs and environmental stressors.
- In the non-adapted killifish population, an interaction between early-life PAH and hypoxia exposure was present. The results indicated that PAH or hypoxia exposure alone significantly decreased swim performance; however, the co-exposure did not generate the same effect. This finding further corroborates antagonism between the PAH and hypoxia mediated toxicity pathways.
- Mitochondrial oxygen consumption, at baseline and at an induced maximal rate, increases with PAH exposure for the non-adapted fish and hypoxia exposure for PAH-adapted fish.

Abstract

Growing evidence suggests that acute polycyclic aromatic hydrocarbon (PAH) exposures have toxic mitochondrial effects and may inhibit aerobic respiration. However, the effect of subteratogenic exposures during development is not well described in literature – especially in the presence of other deleterious environmental conditions. For example, Atlantic teleost fishes experience seasonal hypoxia that may exacerbate co-occurring PAH exposure due to molecular crosstalk with the aryl hydrocarbon receptor (AhR) pathway. This study investigated the potential link between sustained swimming performance and mitochondrial oxygen consumption in two populations of *Fundulus heteroclitus* months after a single initial exposure to a PAH mixture with and without hypoxia. This study used lab-reared embryos from killifish originating in the Elizabeth River (Portsmouth, VA) near a polluted wood treatment facility where the fish have become highly resistant to developmental cardiac teratogenicity (Republic Creosoting; ~113886 ng PAHs/g dry sediment). Another population of killifish were sourced from an undeveloped reference location (King's Creek; ~526 ng PAHs/g dry sediment) outside the Elizabeth River. Subset individuals were treated with either a subteratogenic dilution of a complex PAH mixture ($\sum$[PAHs] ≈ 25.2 μg/L) for 24 hours post-fertilization (hpf), diurnal hypoxia exposure for 14 days post-fertilization (dpf), or both. Upon reaching 6 months of age, their sustained swimming velocity (Ucrit) was measured in a recirculating swim chamber. A separate subset was processed for basal and mitochondrial oxygen consumption rate (OCR) analysis. The study found that killifish population that had historically little PAH exposure had a higher sustained swimming performance than killifish adapted to PAHs. Additionally, mitochondrial oxygen consumption, at baseline and at an induced maximal rate, increases with PAH exposure for the non-adapted fish and hypoxia exposure for PAH-adapted fish.

Background

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic molecules that are comprised of fused aromatic carbon rings. Many chemicals in this class originate as products and byproducts from incomplete combustion reactions. Crude coal tar produced from pyrolysis of coal is one such product that contains a complex mixture of PAHs. While many PAHs have become chemicals of environmental concern due to their nuclear DNA damaging properties, and subsequent carcinogenicity, recent studies have uncovered PAHs to specifically target the mitochondrial genome[1]. For example, aquatic organisms exposed to benzo[a]pyrene are suspected to have bioenergetic deficiencies in terms of mitochondrial respiration that can even perpetuate down the germ line (Kozal, et al., *in prep*).

Near the town of Chesapeake, VA, a well-documented Superfund site has been closely studied for decades to determine the impacts of PAH contamination on the local aquatic ecology. A local estuary of Chesapeake, the Elizabeth River is abundant in timber resources and has served as the setting for many wood coating industrial facilities until the 1990s. One such wood treatment industry that thrived in the area was coal-tar creosote manufacturing. Still widely used today, creosote was the main active ingredient in wood preservative used to defend against marine wood borers. Principle among the wood treatment industries was Republic Creosote which left a long-standing legacy of creosote pollution in the Elizabeth River estuary. Creosote, which is produced from distilling coal tar, is a complex mixture of many different PAHs[2].

The purposeful dumping and leaching of creosote into the Elizabeth River over many decades have shown noticeable health effects in the local aquatic community[3]. Specifically, Atlantic killifish (*Fundulus heteroclitus*) is an estuarine fish with a limited home range in the Elizabeth River system. Their lack of seasonal mobility puts them in direct waterborne exposure

to the PAHs bound to the sediments and organic substrates in the Elizabeth River. Tissue samples collected from these local populations reveal a higher occurrence of neoplasms and other cancer markers[4] than unexposed populations. In addition, many fish suffer bioenergetic ailments that stem from cardiovascular and neurodevelopmental defects that are born from acute and sub-chronic PAH exposures[5].

The molecular mechanisms by which PAHs disrupt mitochondrial respiration in cardiomyocytes are speculated to be numerous but remain poorly characterized. One postulated mechanism involves PAHs activating the aryl-hydrocarbon receptor (AhR). The AhR pathway in turn activates xenobiotic response elements which serve as transcription factors that bind to the promoter regions upstream of genes coding for the cytochrome P450 family[6]. The transcription of cytochrome P450 enzymes (CYP1A1, CYP1A2, CYP1B1, etc.) enable the enzymes to metabolize PAHs through phase I metabolism[7]. Oxidation-reduction reactions performed to increase the water solubility of PAHs routinely produces reactive oxygen species and superoxide radicals that can irreparably damage cellular and mitochondrial machinery. PAHs are also metabolized to toxic diol-epoxides that may covalently bind to guanine nucleobases in DNA and mtDNA. These DNA adducts may eventually lead to improper DNA polymerase binding and transcriptional errors that can affect protein production along the inner mitochondrial membrane.

In addition to PAH exposure, seasonal waves of low oxygen conditions are known to occur in the Chesapeake Bay. Since the 1950s these hypoxic episodes are increasing in frequency and their spatial coverage[8]. Under hypoxic conditions, oxygen can be a limiting factor in energy metabolism. Furthermore, oxygen is the primary regulator for hypoxia inducible factor-1α (HIF-1α). HIF-1α is continuously produced and broken down under normal oxygen conditions (~8 mg/L). Prolyl hydroxylase domain (PHD) proteins are required for the breakdown

of HIF-1α transcription factors, and the activation of these proteins are dependent on oxygen binding[9-11]. At low oxygen concentrations, the activity of PHD proteins decreases and HIF-1α's increase in density. At high densities, HIF-1α's can be translocated to the nucleus of the cell and dimerize with HIF-1β. This beta subunit – also known as the aryl hydrocarbon nuclear translocator (ARNT) – is involved with dimerizing to AhR in the nucleus[12]. These protein heterodimers serve as transcription factors for a variety of genes including those related to energy metabolism. The sequestering of ARNT by the HIF-1 pathway and vice-versa may produce an altered physiological response than if only one pathway is active. However, there is no indication currently if co-exposure to hypoxia and PAHs and potential ARNT sequestration is developmentally persistent.

Bioenergetic markers are a useful tool in studying the effects of PAH, hypoxia, and their combined interaction on organismal development – although few studies have attempted to take on such extensive exposure scenarios[13,14]. In this study *F. heteroclitus* serves as a model to study how the co-exposures of PAHs and hypoxia during development will have health effects at later life stages. Previous studies have examined how PAHs can specifically target and localize in the mitochondria, but there is a notable shortcoming in the current literature that seeks to understand the effects of complex PAH exposures along with secondary physical exposures on whole-organism bioenergetics[15]. In addition, effectively only a single study has followed killifish throughout development with the focus of measuring bioenergetics in later life stages after a single instance of exposure[16].

To investigate the potential role PAH pollution adaptation had on the long-term effects of embryonic exposure to PAHs and hypoxia, two populations of killifish were exposed to similar exposure conditions. Wild-caught killifish were brought into the lab from a contaminated site in

the Elizabeth River, Va (Republic Creosoting) and a reference site (King's Creek). Manually spawned killifish embryos from Republic and King's Creek sites were exposed for 24 hours after fertilization to a complex mixture of PAHs taken from anoxic pore water from the Atlantic Woods Superfund Site. After exposure to this Elizabeth River sediment extract (ERSE), hypoxia treatments then started and lasted for 14 days post-fertilization. Embryos are thus grouped in a total of four different treatment groups consisting of all possible exposure permutations (normoxia and 0% ERSE, normoxia and 0.5% ERSE, hypoxia and 0% ERSE, hypoxia and 0.5% ERSE). To maintain hypoxic conditions over time, exposure was tightly and continuously measured by an oxygen probe and readjusted when necessary by nitrogen injections into the incubators. After 6 months of development, these fish were then dissected, and their mitochondrial respiration in specific tissue samples was measured. Mitochondrial oxygen consumption assays along with sustained swimming trials were used to determine if early life exposure to environmental contaminants may influence behavior and bioenergetics later in life.

Objectives

The objectives of this project are to a) understand how co-exposures of hypoxia and PAH impact mitochondrial function and bioenergetics in killifish and to b) ascertain whether PAH-resistant populations of killifish are more metabolically vulnerable to co-exposures compared to reference killifish.

These objectives were accomplished through four primary approaches:

1. Expose killifish embryos throughout development to hypoxic or normoxic conditions, with and without non-teratogenic doses of ERSE

2. Measure metabolic rate via oxygen consumption and swim performance after 6 months of development in pre-exposed killifish populations using a swim tunnel respirometer
3. Measure tissue-specific alterations in mitochondrial function using the Seahorse Extracellular Flux Analyzer
4. Assess data for statistical differences in whole-organism bioenergetics and metabolic rate between treatment groups across the two fish populations

Methods

Breeding and Embryonic Exposures

Adult killifish were collected from a site near the defunct Republic Creosoting (36°47'39.65" N, 76°17'31.94" W) manufacturing plant in the Elizabeth River (VA, USA) in the summers of 2017 and 2018. Reference killifish were sourced outside of the Elizabeth River at Kings Creek (37°18'16.2" N, 76°24'58.9" W) which has negligible PAH concentrations in sediments. Eggs and sperm were collected from wild-caught adult fish and given one hour to complete fertilization. At 24 hpf embryos were screened for viability and developmental stage. Appropriately developing embryos were then exposed in glass scintillation vials to either Danieau's solution or 0.5% Elizabeth River Sediment Extract (ERSE). A subsequent exposure to diurnal hypoxia (for fish exposed and unexposed to 0.5% ERSE) then took place. Exposures lasted for 14 days, until hatching.

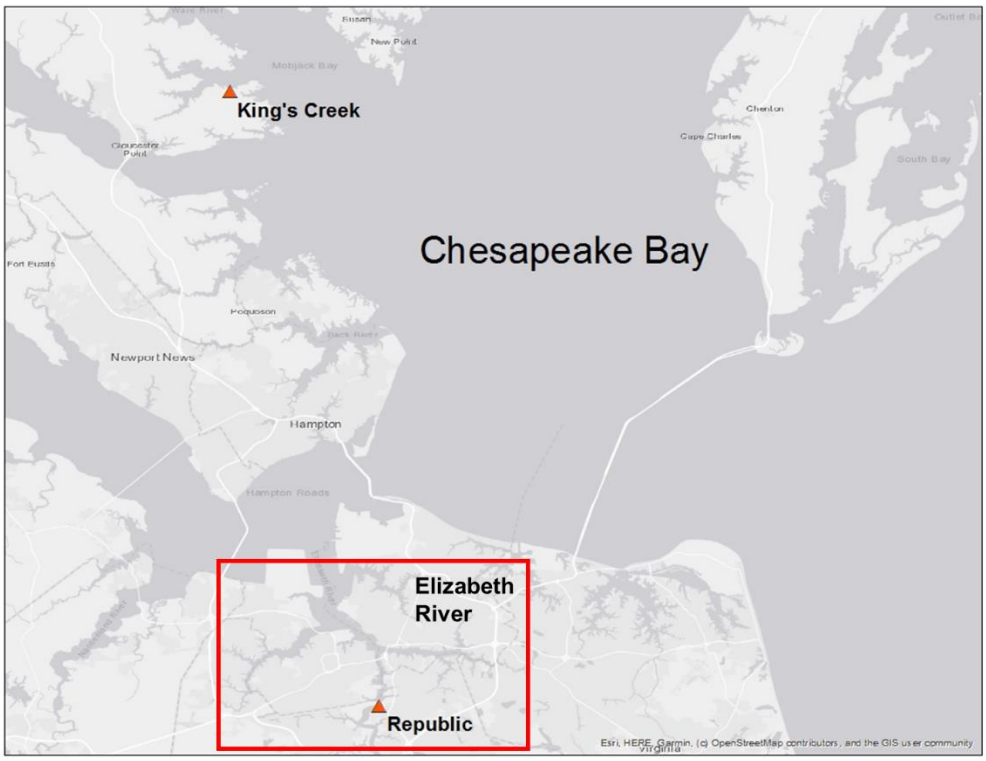

Figure 1. Map of the Chesapeake Bay, VA with Elizabeth River outlined in red. Republic and King's Creek reference site are plotted. The King's Creek reference is within the same local watershed but far removed from any PAH pollution bound to Elizabeth River sediments.

Hypoxia exposures were conducted in a Heraeus Heracell 160i Tri-Gas Cell Culture Incubator (Thermo Scientific, Waltham, MA) maintained in isothermic conditions (28°C) and a light cycle (14:10 light:dark) that is identical that is identical to laboratory fish raised in our aquatic habitat system. Hypoxia exposures occurred for 8 continuous hours during the day (10am-6pm every day) for 14 days at 5% oxygen saturation in ambient air (~2.5 mg/L). Normal ambient air conditions were controlled at 21% oxygen (~8 mg/L). Oxygen adjustments were accomplished by injection of nitrogen into the incubator and was monitored and regulated constantly by an oxygen probe throughout the diurnal exposure period. After sealing the incubator, oxygen concentrations in the vials equilibrates with the air in the chamber after approximately 6-8 hours[17].

Embryos that underwent diurnal exposure to hypoxia occurred for 2 weeks (14 dpf) starting at approximately 32 hpf. An 8-hour exposure limit was chosen for the studies because it easily mimicked the length of natural estuarine exposures. In many estuaries, hypoxic conditions only occur at night (~8 hours) and are quickly alleviated with primary production during the day[18].

Fish Care and Maintenance

Broodstock from the Republic and King's Creek sites were transferred to separate chambers in the same recirculating aquatic habitat systems (8 tanks with approximately 5-10 fish per tank) in the Duke University Ecotoxicology lab. Spawned embryos were collected after 1 hour of noninduced spawning following Duke IACUC animal handling protocols.

Two weeks post-hatching, killifish fry were placed in aquarium tanks by exposure and population group. During this time fish were fed twice daily with particles <50 microns from Zeigler's dry larval fish diet (Zeigler Bros, Inc., Gardners, PA). Approximately, 200 mL of 15ppt artificial seawater (ASW) (Instant Ocean, Foster & Smith, Rhinelander, WI, USA) was added to each tank after every feeding to maintain water clarity and temperature. After maturing an additional two weeks, surviving fry were placed in a recirculating aquatic habitat system (AHAB) at a consistent 25-28°C and 14:10 hour light:dark cycle. The habitat system was maintained at a salinity between 10-15ppt ASW during the maturation period. Nitrite, nitrate, and ammonium levels were checked on a weekly basis. Juvenile killifish were not directly handled for at least one month, and tank cleaning was accomplished through syringe removal of particulates, mold, and floating debris. After one month, fish were placed in groups of 3-5 by exposure and population to promote equal growth and to prevent overcrowding in the tanks.

The Duke University Institute for Animal Care and Use Committee approved all the handling and experimental techniques described below (IACUC Protocol #: A139-16-06)

Chemicals

ERSE is a complex mixture of PAHs that was originally taken from the Atlantic Wood superfund site in the Elizabeth River (36°48′27.2″N, 76°17′38.1″W). This complex mixture contains both AhR agonists and non-binding ligands whose effects may be dependent on other compounds in the mixture. The total sediment burden of PAHs at the contaminant and reference sites, characterization of the PAHs in ERSE, and the fractionation procedures for the porewater water extracts are detailed in previous literature (Clark et al. 2013 and Fang et al. 2014)[19,20].

The Ringer's solution was created using buffers and concentrations adapted from Seebacher et al. (2015)[21] The mixture consists of HEPES buffer solution, sodium pyruvate, and a series of electrolytes including sodium bicarbonate, sodium chloride, potassium chloride, magnesium chloride, monosodium phosphate, and calcium chloride. HEPES buffer is an ideal buffering solution to ensure that the release of CO_2 will not impact the integrity of the cells during the Seahorse trials. Unlike many other buffers used to sustain cell cultures, HEPES is stable at physiological pHs even under circumstances where there is a changing concentration of carbon dioxide[22]. This is an ideal buffer because the pharmaceutical agent that elicits maximal mitochondrial respiration, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), may invoke a spike in carbon dioxide concentrations while electron transport inhibitors will decrease CO_2 levels.

The pharmaceutical agents FCCP, antimycin A, and rotenone are the principal drugs used to evaluate oxygen consumption rate in the heart, gonadal, and brain tissues. FCCP induces

maximal respiration in the targeted cells by shuttling protons along their electrochemical gradient back into the mitochondrial matrix[23]. Electron transport mechanisms along the inner mitochondrial membrane will then accelerate to maintain the electrochemical gradient. Antimycin A is a crystalline antibiotic and known piscicide while rotenone has typical applications as a broad-spectrum pesticide. Both agents target the mitochondria by inhibiting proteins along the electron transport chain. Rotenone is a potent complex I inhibitor while antimycin A concurrently inhibits complex III. Their injection effectively halts oxidative phosphorylation and mitochondrial ATP production.

Killifish Sustained Swim Performance

After juvenile King's Creek and Republic killifish reached 6 months of age, they underwent swim performance assessment in a controlled swim tunnel (Loligo® Systems, Viborg, Denmark) environment. Killifish from each group (n=8-11) were taken and swam individually, in the same recirculating swim tunnel, across multiple days. Individual killifish were placed into the enclosed swim chamber (containing 10-15 ppt ASW) with a respirometer and allowed to acclimate for 1 hour. Oxygen levels were recorded in real time by a PreSens O_2 respirometer and visualized on PreSens' OxyView software (PreSens GmbH, Regensburg, Germany). During the acclimation period water was circulated from the reservoir tank into the swim chamber so that the fish had plentiful access to oxygen.

Once fish were acclimated to the tunnel and the respirometer showed no large fluctuations in dissolved oxygen level, basal metabolic rate (BMR) measurements were taken by halting the water flow into the swim chamber and calculating the difference in oxygen levels within the chamber across a 5-minute period. During the recording period, the fish was observed to ensure

no erratic behaviors were occurring that may skew baseline measurments. After the initial recording period, water flow was resumed to reoxygenate the chamber for 10 minutes. In total, this process was repeated until five BMR measurements were taken. The minimum value was taken as the standard metabolic rate (SMR) or the whole-organism oxygen consumption rate at rest.

Swim performance assessments were conducted immediately after SMR calculations. To measure swim performance, the critical maximum swimming speed prior to exhaustion (U_{crit}) was found following protocols established by Brown et al., 2017[24]. In these protocols, killifish swam in water velocities that are a function of their body lengths (BL) and incremental adjustments to the water velocity were made every ten minutes. Adjustments to water speed were made by increasing the RPM of a motor attached to a fan upstream of the sealed swim tunnel. Prior to the test, fish swam for 30 minutes at 0.5*body length(cm)/sec to initiate positive rheotaxis with the current. Afterwards fish swam from 1*BL to exhaustion, where the water velocity increased by 0.3 BL after every 10 minutes. Once the fish had reached exhaustion, the fish was swept by the current to the downstream end of the swim tunnel guarded by a baffle. If the fish could not unpin itself from the baffle after 10 seconds, the fan was shut off and the water flow stopped. If the fish could not swim in the current speed after three attempts, the test ended. The final time and speed was used to calculate U_{crit}. The full formula for U_{crit} is calculated identical to Hammer et al., (1995)[25]:

$$\mathbf{U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]}$$

where U_i is the final water velocity (BL (cm)/sec), U_{ii} is the water velocity increment at each step (0.3*BL/sec), T_i is the time elapsed at the final velocity when the fish reached exhaustion (min), and T_{ii} is the interval time between incremental water velocity steps (min).

The maximal metabolic rate (MMR) was considered to be the rate at which fish consumed oxygen after being consecutively pinned to the baffle. After stopping the fan and allowing the fish a few seconds to remove themselves from the baffle, MMR was measured immediately. The swim chamber was cut off from the recirculating reservoir water, and the oxygen was allowed to be consumed without replenishment. Dissolved oxygen concentrations were measured every minute for five minutes. Only the maximum oxygen consumption rate observed of these five measurements was recorded as the MMR.

The data from these tests were analyzed under a 3-way analysis of variance (ANOVA) and Tukey's post-hoc test. Upon creation of the full model, model fitting techniques – Akaike Information Criterion (AIC) and stepwise regression (AIC and R^2) – were used to assure the model best fits the data. The best fit model was used to determine if any significant differences oxygen consumption and critical swim velocity exist between established treatment groups. The best fit model was tested for independent and identically distributed datapoints for parametric ANOVA assumptions. Tests for the normality and homogeneity of variances found the model data to be approximately normal and homoscedastic in the case of Ucrit. Metabolic measurements were normalized by total wet weight and negative natural log transformed to meet normality. The transformed data was subsequently used in parametric hypothesis testing.

Mitochondrial Respiration

To determine differences in metabolic responses in juveniles that developed under embryonic ERSE and sub-chronic hypoxia exposure, the juvenile fish were dissected after swim performance assessments for heart and brain tissue samples. Prior to each dissection, killifish were shocked in ice water (taken from the killifish AHAB) and then euthanized via cervical

spine dislocation. Tissue extraction was conducted in groups of 7 fish and took approximately 1 hour between the first dissection and plating the last tissue in Ringer's solution. Brain tissue was collected by scraping along the skull plate and plating whole cerebrum or sample slices in the case whole tissue exceeded 10 mg wet weight. To extract cardiac tissue, an incision was made along the ventral side from the anal fin to the mouth of the fish. Hearts were then clamped by the bulbus arteriosus and excised through the ventral cavity. Extracted tissues were immediately weighed and stored within a Seahorse XF24 Islet Capture Microplate (525 mL Ringer's solution per well) before moving on to the next dissection. All plates had at least three blanks with Ringer's solution alone. After all tissues were successfully plated, screens were placed in each well to contain the tissue sample at the bottom center position of the well.

The XFe24 Seahorse Extracellular Flux Analyzer (Agilent, Santa Clara, CA, United States) was then used to measure basal, maximal, and mitochondrial respiration of brain and cardiac tissue samples – the processes of which are well described in literature[26]. Prior to oxygen consumption measurements, the internal incubator was set to 28 C (27.8-29.0 C in actual due to the machine running hot). Tissue-specific basal oxygen consumption rate (OCR) followed established protocol by Jayasundara et al., 2015[27]. Eight injections of dissolved FCCP (75 µL total) in Ringer's solution was applied every 3 minutes following a two-minute wait after basal oxygen consumption measurements. Followed by FCCP injections was a two-minute wait period followed by 25 successive injections (once every 3 minutes) of antimycin A and rotenone jointly (75 µL of mixed solution) dissolved in Ringer's solution. The additive effect of the two chemicals completely inhibits the oxidative phosphorylation ATP synthesis pathway and allows for the measurement of background oxygen consumption.

An average of the three lowest, highest, and lowest measurement points were used to determine representative OCRs at baseline, post-FCCP, and post-Antimycin A + rotenone respectively. Basal OCR was calculated as the difference of OCR post-Antimycin A + rotenone from baseline OCR. Maximal OCR is the difference of OCR after Antimycin A and rotenone injection from post-FCCP. The respiratory aerobic scope is the difference between OCR pre and post-FCCP injections.

Three-way ANOVA after model fitting were used to determine if any significant differences in basal, maximal, and mitochondrial respiration exist across PAH and hypoxia exposure, population, and other relevant parameters such as wet weight and cohort group. Mitochondrial oxygen consumption was averaged before and after each drug treatment and the consumption rate for each tissue was normalized by the sampled tissue weight.

Results

Swimming performance

Wet weight (g), body length (mm), height (mm), and width (mm) were measured for each fish prior to acclimation in the recirculating swim tunnel. Fish were handled by capturing them with a small net and placing them out of their tank onto a dissection tray. Fish were immediately covered by placing a hand slightly above the fish. After ~5-10 seconds when the fish becomes calm, body proportions were quickly measured using calipers. Fish were then weighed and placed into the oxygenated swim chamber.

Weight is of particular interest because it is typically confounding as larger fish are generally found to have higher swim stamina relative to their body lengths[28]. It's unclear if hypoxia and ERSE exposures may promote adipogenesis select for fish with higher lipid stores,

so little information is known if weight increases lie on the causal pathway between exposure and swimming performance. However, because the growth and juvenile size of a killifish is dependent on many factors unrelated to exposure, such as stocking density, the Ucrit linear models herein must control for the effect of wet weight (mg) measured immediately before swimming tests. Weight was not significantly different across treatment groups or populations; however, wet weight (mg) became a significant predictor for Ucrit once added to a general linear model of (ANCOVA in this case) and increases the penalized R^2 marginally (~0.02). Using Bayes Information Criterion (BIC) in stepwise regression model fitting, the variable can be dropped entirely from the true model; however, AIC model fitting keeps the parameter. Due to potential underfitting, wet weight remained in the model. Cohort grouping variable was not significant in the model, so no batch effect was observed.

Table 1. Replicate size (N) and body measurements for killifish included in the swim performance assay for Ucrit. A 3-way ANCOVA with population, ERSE, and hypoxia and predictors showed no statistically significant differences across treatment groups for body proportions. * = significant predictor for Ucrit in linear model.

	N	Wet Weight (mg)*	BL (cm)
King's Creek			
Control	11	796 ± 68	40 ± 1.3
ERSE	10	1010 ± 74	43.3 ± 1.0
Hypoxia	9	936 ± 121	41.7 ± 1.5
ERSE + Hypoxia	11	896 ± 112	40.8 ± 1.6
Republic			
Control	10	998 ± 85	44.2 ± 1.0
ERSE	9	937 ± 128	44.1 ± 2.1
Hypoxia	10	968 ± 164	43.3 ± 1.7
ERSE + Hypoxia	8	1018 ± 198	44.1 ± 2.7

A 3-way parametric ANCOVA shows that population origin has a significant effect on Ucrit. A Tukey's honest significant difference post-hoc test revealed that King's Creek killifish significantly sustained higher swimming velocities than Republic killifish in the swimming performance assay (Figure 2; $p < 0.05$). In addition to a population effect, there was a significant ERSE and hypoxia interaction for King's Creek fish but not Republic killifish. Unexposed King's Creek fish had a significantly increased Ucrit versus hypoxia treatment conditions and significant increase over ERSE exposed fish ($p < 0.05$). The results indicate that King's Creek killifish sustained swimming speed are negatively impacted by hypoxia and ERSE exposures;

however, this difference is obscured by a significant interaction effect. Republic killifish treated fish showed no significant difference for Ucrit compared to the control group.

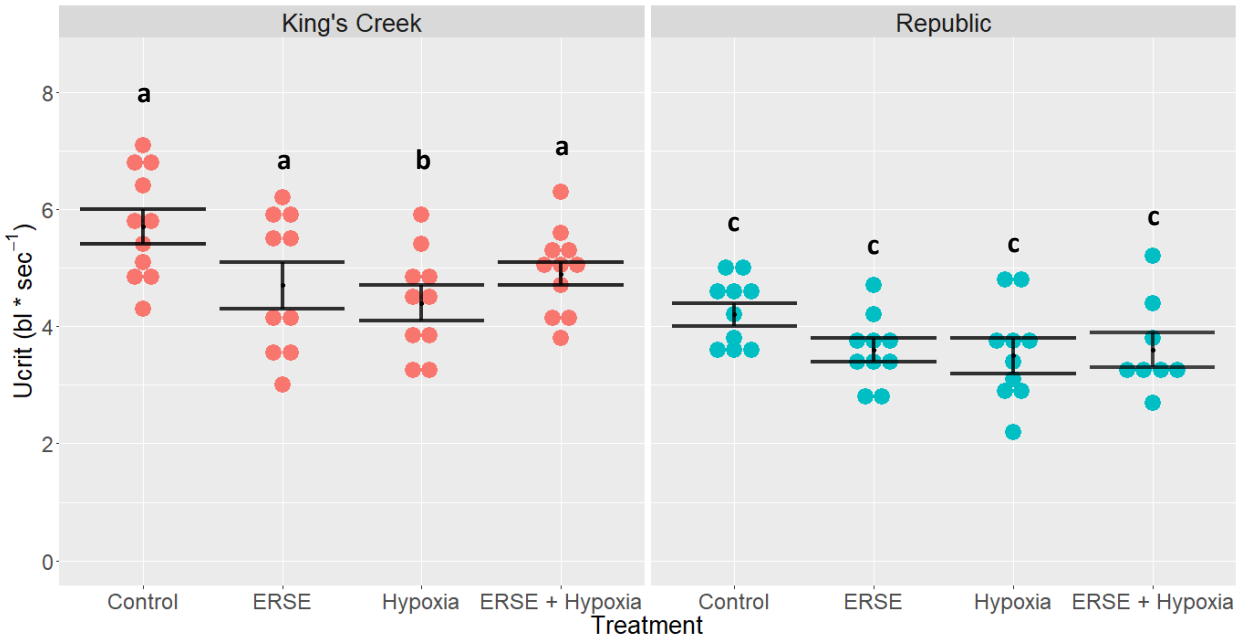

Figure 2. Maximum sustained swimming velocity (Ucrit) for King's Creek (KC) and Republic Creosoting (Rep) killifish six months after embryonic treatment conditions in an incremental swim velocity test. Dot plots have error bars for mean Ucrit values (body length/s) ± standard errors for King's Creek and Republic killifish across each treatment. Treatment groups consist of 0.5% Elizabeth River sediment extract (0.5% (v/v ERSE/Danieau solution) 24 hours post-fertilization, hypoxia (~2.5 mg/L oxygen) for 14 days post-fertilization, and a co-exposure scenario. Each treatment group includes a number of replicates described in Table 1. Letters indicate significant difference among groups.

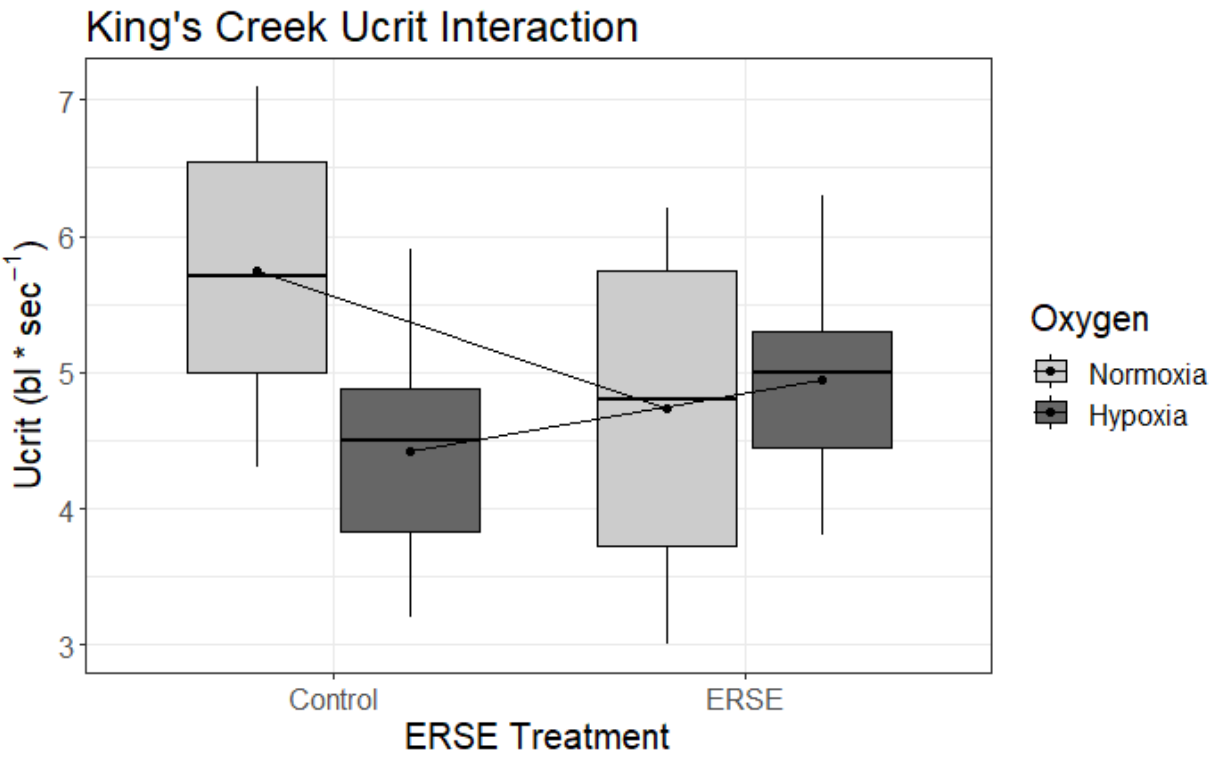

Figure 3. ERSE and hypoxia interaction effect on sustained swimming speed for King's Creek killifish 6 months after single embryonic exposure. Ucrit decreases for fish raised in normoxic conditions when ERSE exposure also occurs. However, killifish raised in hypoxic conditions and exposed to ERSE have an increased Ucrit relative to fish raised in hypoxia alone. Points signify mean values with lines showing the change in mean Ucrit with ERSE exposure.

Oxygen Consumption Before and After Swim Assessment and Aerobic Scope

Residuals were not normally distributed for the ANOVA model, so a data transformation was required to use parametric statistics to test if oxygen consumption rates differed across populations and treatments. To transform the data in order to meet normality of residuals, a negative natural log [-ln(Normalized SMR)] transformation was used. After transformation the data was approximately Gaussian and had independent and identically distributed (iid) residuals. Parametric ANOVA after model fitting revealed that there was no statistically significant difference across populations or treatment groups (Figure 3; $p > 0.05$).

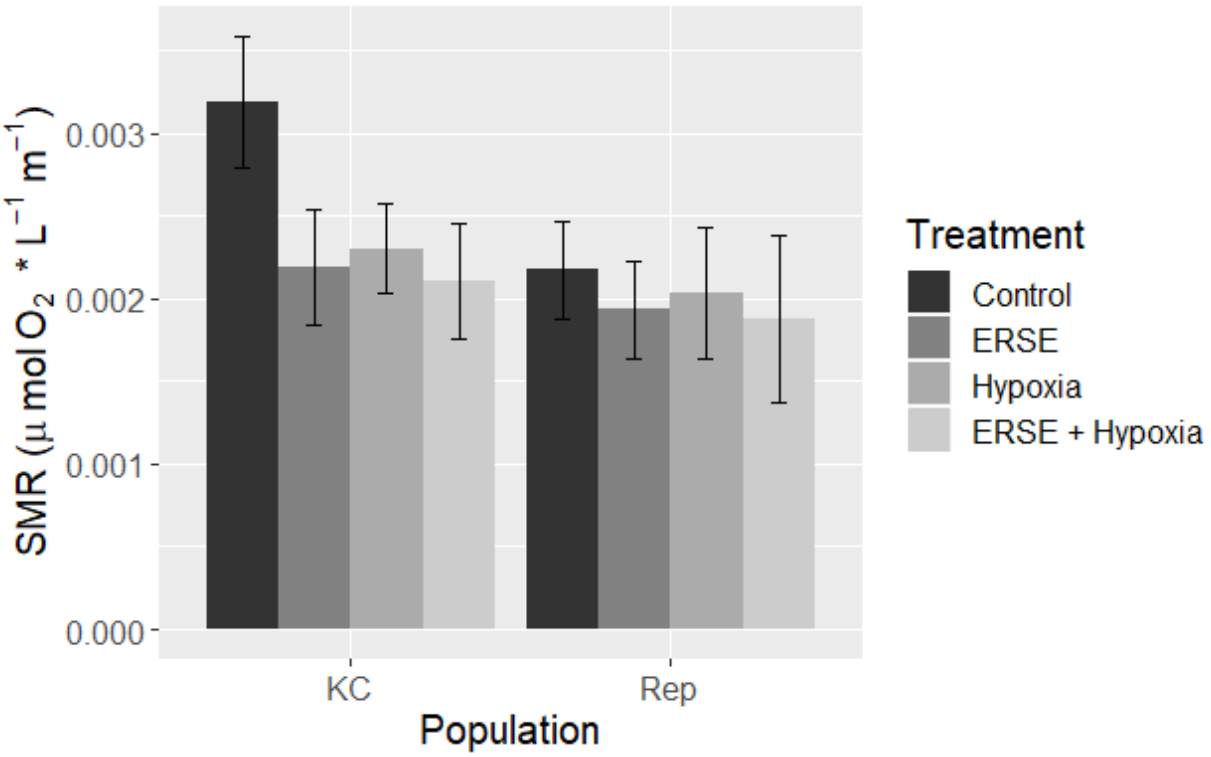

Figure 4. Oxygen consumption at baseline normalized for body weight before Ucrit assays for King's Creek (KC) and Republic (Rep) killifish. Mean oxygen consumption (μmol O_2/L^{-1}/min $^{-1}$) ± standard error for killifish after swim tunnel acclimation and handling stress. No significant differences among populations or treatments in oxygen consumption were found using 3-way ANOVA. Sample sizes for metabolic rate measurements were identical to the sustained swim performance trials.

Maximal oxygen consumption, recorded immediately after every swim performance assay, also needed to be transformed to establish normal residuals. The same data transformation techniques were used for normalized MMR and aerobic scope measurements. Aerobic scope, the difference between the maximal and standard metabolic rates, was calculated to determine the respiratory spare capacity that an individual killifish may have during routine functions. No significant results were found for either response variable (Figures 4 and 5). In addition, no population effect was observed.

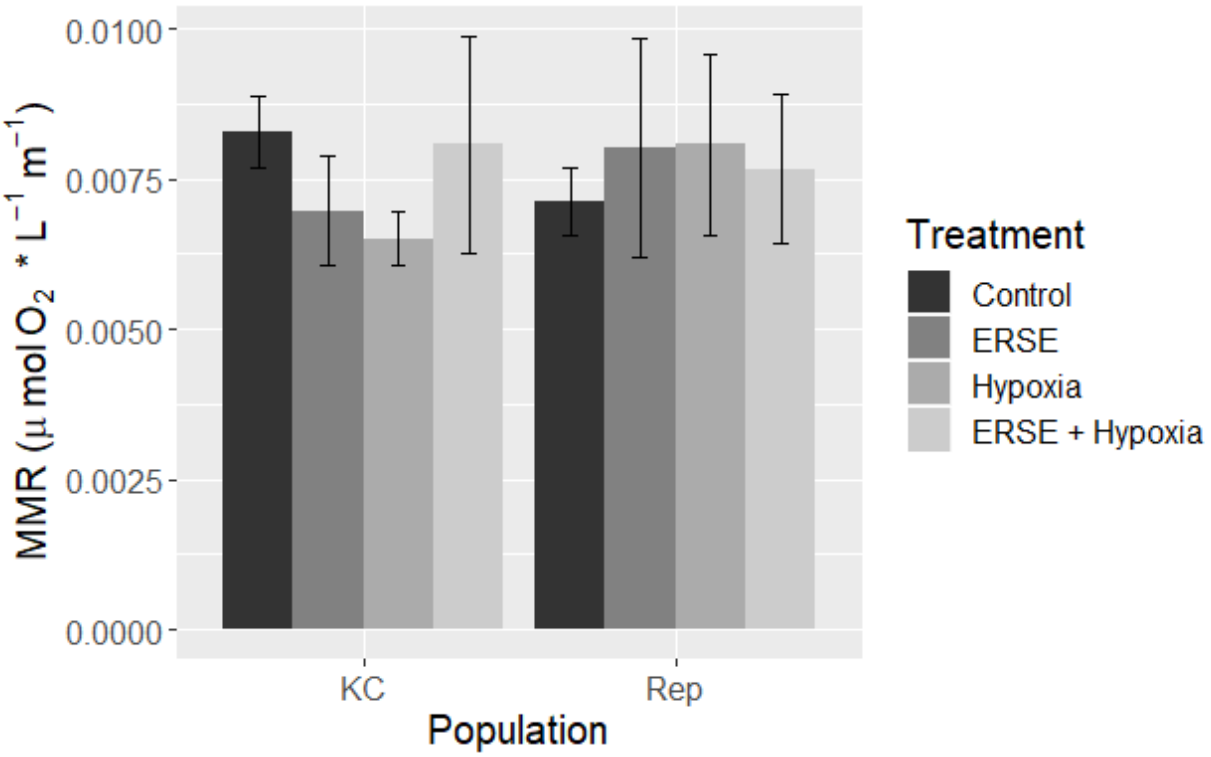

Figure 5. Maximum oxygen consumption normalized for body weight after Ucrit assays for King's Creek (KC) and Republic (Rep) killifish. Mean oxygen consumption (μmol O_2/L^{-1}/min $^{-1}$) ± standard error for killifish after individuals became pinned to the swim chamber baffle three consecutive times in a 10-minute period. No significant differences among populations or treatments in oxygen consumption were found using 3-way ANOVA.

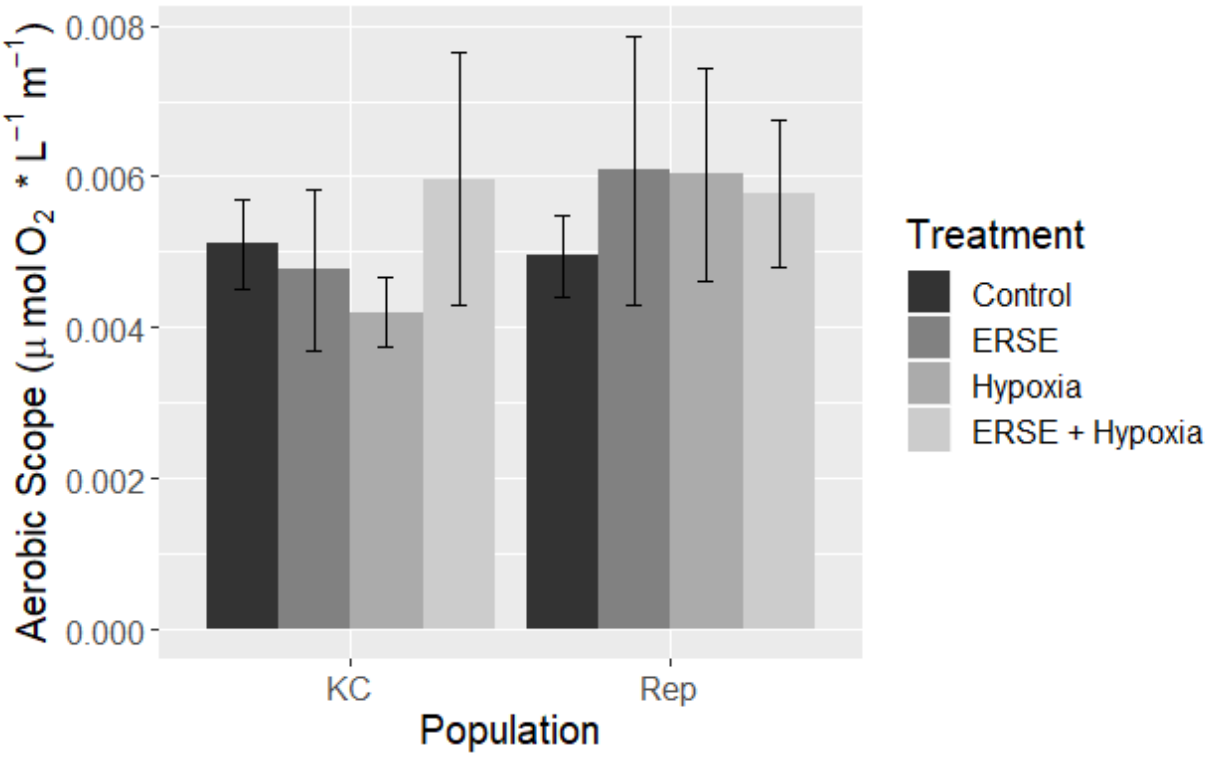

Figure 6. Aerobic scope measured as the difference in per minute oxygen consumption pre and post-Ucrit assays for King's Creek (KC) and Republic (Rep) killifish. Mean oxygen consumption (μmol $O_2/L^{-1}/min^{-1}$) ± standard error for killifish. No significant differences among populations or treatments in oxygen consumption were found.

Mitochondrial Oxygen Consumption Rate

Only basal measurements were recorded for juvenile killifish brains. FCCP was not able to be optimized for the brain; however, Antimycin A and rotenone injections were still positive in lowering OCR. Fundulus hearts were able to be optimized for FCCP, and FCCP was dissolved directly in Ringer's solution without centrifugation. Juvenile hearts received eight injection of 5 μM FCCP after baseline OCR measurements.

Due to differences in survivorship – sample sizes were unequal across treatment groups (KC Control = 9, KC ERSE = 15, KC hypoxia = 11, KC ERSE+hyp = 18, Rep Control = 12, Rep ERSE = 11, Rep hypoxia = 11, Rep ERSE+hyp = 5). Basal and maximal OCR in hearts

increased in response to ERSE exposure for King's Creek killifish, but not Republic. Inversely, basal and maximal OCR was marginally elevated ($p = 0.07$ for both) in hearts for hypoxia exposed Republic killifish but not King's Creek fish. No significant difference was found across treatments or populations for brain samples. An interaction between ERSE and hypoxia exposure was not observed (Figure 7).

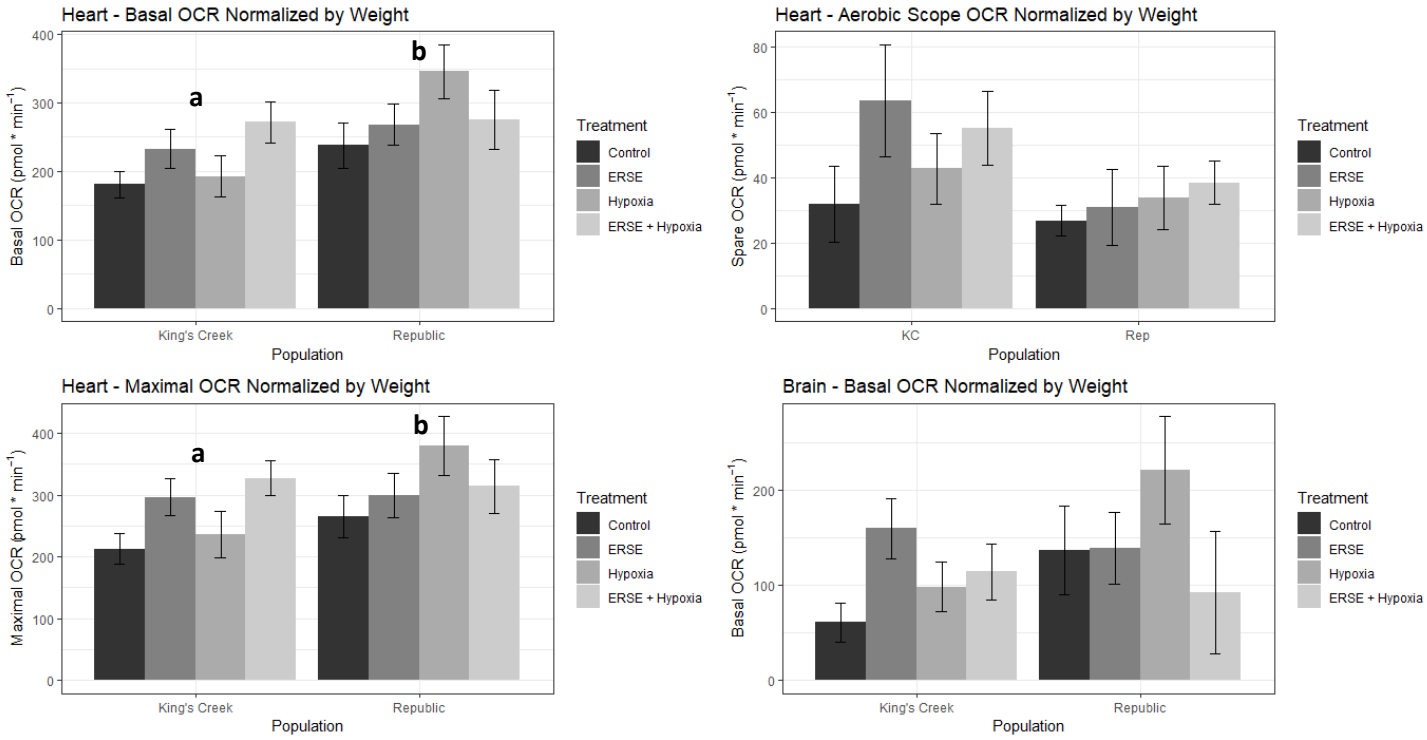

Figure 7. Mean mitochondrial oxygen consumption rate (pmol O_2/min $^{-1}$) with standard error bars for heart and brain tissues extracted from juvenile King's Creek and Republic killifish. Top left: oxygen consumption at baseline for juvenile heart tissues normalized by heart weight. Bottom left: oxygen consumption after FCCP induced proton leak. Top right: difference between maximal and basal mitochondrial oxygen consumption which is the spare or reserve capacity that the mitochondria may work to consume oxygen. Bottom left: basal oxygen consumption levels for juvenile killifish brains normalized by weight. Tukey's HSD showed no significant differences across groups, but ERSE was significant for King's Creek basal and maximal OCR when pooled from both single and co-exposures ($p < 0.05$). Hypoxia was marginally significant Republic basal and maximal OCR when pooled from both single and co-exposed treatment groups ($p = 0.07$).

Discussion

The results from this study are mixed with regard to the swim performance. For King's Creek fish, linear models with interaction show that 0.5% (v/v) ERSE and diurnal hypoxia exposures decreased the mean Ucrit significantly relative from controls. However, a two-way post-hoc test within populations does not distinguish specific differences between ERSE exposed fish with and without hypoxia exposure. This finding is significant because it suggests that simultaneous PAH and hypoxia exposure may be protective compared to a single exposure. In

addition, ERSE and hypoxia exposure in Republic killifish were not able to elicit the same impairment in swimming speed as seen in the King's Creek population – indicating that an evolutionary adaptation to PAH mediated toxicity may further be protective in the case of early acute exposures to PAHs. Although power tests indicate that the current sample sizes are adequate for hypothesis testing given the number of treatment groups, the sample sizes are too limited to determine the true effect of treatment on sustained swimming speed. Further testing will need to be done to determine the validity of a protective mechanism and if the protective effect is unidirectional in nature.

Many factors lying between the causal pathway of early embryonic exposure and later life Ucrit outcome may help explain the variation seen across populations and treatment conditions. This study focuses on both whole-organism respiration and mitochondrial oxygen consumption at basal and maximal levels to determine if there was any significant alteration on the respiratory spare capacity that may predict Ucrit. Whole-organism respirometry measurements for treated King's Creek and Republic killifish failed to capture any significant differences for SMR, MMR and aerobic scope within treatment groups or populations. These metabolic rate measurements are conflicting in recent literature. In a study comparing swim performance in juvenile mahi-mahi, Mager et al. (2014) found that SMR, MMR, and aerobic scope (scaled to a standard mass of 1g) did not differ across exposures to increasing concentrations of Deepwater Horizon crude oil mixtures[29]. Brown et al. (2017), however, found opposing results in a study that compared sustained swimming speed in King's Creek killifish and Atlantic Wood killifish (PAH sediment loading comparable to our Republic site) exposed to ERSE during embryonic development. That study found mean oxygen consumption was higher at baseline for untreated Atlantic Wood killifish and King's Creek fish treated with 0.1% and

1.0% ERSE compared to untreated controls. While whole-organism oxygen consumption (normalized by body weight) did not significantly differ across population and treatment conditions a subcellular approach investing mitochondrial based oxygen consumption revealed altered O_2 uptake rates.

Mitochondrial oxygen consumption showed a trend opposite of what was observed in the Ucrit tests. Mitochondrial oxygen consumption in cardiac tissue has a population level effect in which Republic killifish tissues consume more oxygen at basal and induced maximal levels. In addition, King's Creek heart samples from fish exposed to ERSE have elevated OCR relative to controls. These findings are in line with the Brown et al. (2017) study, but the same trend is not observed for hypoxia exposed King's Creek fish. In Republic fish, cardiac OCR significantly increases for fish exposed to hypoxia but not ERSE, indicating that PAH adaption provides some physiological resilience for early life exposure, but sacrifices some resilience to other types of environmental stressors.

A study conducted by Jayasundara et al., (2017) found similar findings in *Fundulus heteroclitus* cardiac tissue[30]. A comparative bioenergetics approach using the same methodology as this study investigated mitochondrial OCR of wild-caught killifish from King's Creek and the highly contaminated Atlantic Woods site. In the absence of acute thermal stress, killifish from the Atlantic Woods site had significantly increased cardiac OCR at basal and maximal metabolism. Furthermore, omics studies provide evidence that a shift in mitochondrial phenotypes that favors increased oxygen uptake is indeed likely for Elizabeth River killifish[31]. While an increase in OCR was not hypothesized, it stands to reason that there are benefits for this unexpected bioenergetic shift. Killifish adapted to PAHs, or exposed early to some environmental stressor, need greater amounts of ATP to power stress response pathways. ATP-

linked oxygen consumption was not included into the scope of this study but would be a useful approach for determining if there is an increased demand for ATP in Elizabeth River killifish.

While the increase of mitochondria driven oxygen consumption after toxic environmental exposure has been previously described, single early exposure has not been rigorously studied. Previous studies that measured citrate synthase and cytochrome c oxidase activities in *Fundulus heteroclitus* one month after intermittent diel hypoxia exposures observed elevated mitochondrial capacity in the liver (Borowiec et al., 2015[32], Du et al., 2016[33]). Interestingly, further studies demonstrated that augmented mitochondrial respiration from intermittent hypoxia acclimation may be heritable – suggesting that later-life altered mitochondrial function can result from a sub-chronic instance of early life exposure (Du et al., 2016[34]).

The trends observed at the basal level in cardiac tissues were not reflected in killifish brain tissue. Diffuse brain hypoxia is of particular interest in the case of early life exposure because prolonged oxygen deprivation and subsequent impairment of cerebral function in early life may cause irreparable changes in glycogen metabolism and motor control function[35,36]. However, the trends remain similar. ERSE and hypoxia exposure have a non-significant positive effect on King's Creek killifish basal OCR, but the co-exposed treatment group is in between the two. Conversely, Republic killifish have a non-significantly higher basal OCR from embryonic hypoxia exposure, but the response from ERSE exposure is the same as controls. Republic killifish adaptation to elevated PAHs in sediments may blunt the effect of acute PAH exposure early in life, but more samples are needed to show that there might be a real effect. Killifish reared from the King's Creek reference site have no generational history with PAH exposure – providing some explanation as to why they might be affected by early acute exposure to PAHs. Interestingly, early acute hypoxia exposure may not affect King's Creek fish in the same manner

as Republic fish, suggesting that fish embryos nonadapted to PAHs may have greater tolerance to other physical or environmental stressors such as hypoxia.

While interesting, the effects seen in this study may be impacted by confounder variables; whether variables such as weight lie outside the causal pathway of PAHs and hypoxia remains highly questionable. PAH exposure may select for fish with higher lipid profiles to mitigate the risk that PAHs may bind to critical cellular machinery[37]. In addition, PAHs and intermittent hypoxia are believed to influence lipid metabolism[38,39]. Weight and developmental timing, however, are also factors of tank stocking, and tanks with fewer stocked tanks may have induced more rapid growth in certain treatment groups. For example, embryos from the Republic killifish co-exposed to ERSE and hypoxia had the lowest overall survivor rates and thus had fewer fish per tank (~2-3 fish/L) compared to some tanks that were stocked with 5 to 6 fish. Additionally a previous study indicated that *Fundulus Grandis* are aggressive to their own species during growing periods which further affects survival rates (Ofori-Mensah et al., 2018[40]). The consequences of this auto-selection are a strong possibility of survivor bias for juvenile killifish undergoing Ucrit assessment. The optimum density for rapid development of Gulf killifish in a recirculating aquatic habitat system was reported to be 2 fish/L. Due to space limitations, killifish groups with relatively high survivorship were stocked in tanks with upwards of 6 fish which may have played a role in sustained swimming performance later in life. In addition, killifish survivability in the King's Creek population favored control groups despite similar stocking densities across treatments. This may indicate that treated fish may exhibit more aggression than control fish and introduces a selection bias.

Conclusion and Summary

Despite conflicting mechanistic studies and underlying sources of error, the findings support the hypothesis that early life exposure of an ecological fish model to ERSE and hypoxia lead to decreased swim performance later in life. These results are suggested to be partially representative of altered mitochondrial physiology later in life. A comparison of the results between King's Creek sustained swimming performance and mitochondrial OCR shows that cardiac mitochondrial capacity (basal or maximal) does not provide a clear explanation for locomotor performance at the whole-organism level. It's worth noting that samples in this study have wide variations in response, perhaps due to wide genetic diversity of the killifish cohorts (even within populations), the complex nature of the Elizabeth River sediment extract used to simulate environmentally relevant PAH exposures, and the developmental timing of fish in different stocking situations. Nevertheless, our study presents another perspective for how hypoxia may interact with the AhR mediated toxicity pathway in unexpected ways, and that pollution driven adaptation may impart physiological differences in dealing with other environmental stressors. In light of this study, and the recent literature, there is a critical need to further examine the immediate and lasting effects of acute PAH and hypoxia exposures for both adult and embryonic organisms.

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