Later-life effects of early-life benzo(a)pyrene and triphenyl phosphate exposure on respiration in zebrafish (*Danio rerio*)

by

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ABSTRACT

Benzo(a)pyrene (BAP) is a polycyclic aromatic hydrocarbon with carcinogenic metabolites. Triphenyl phosphate (TPP) is an organophosphate ester often used as a flame retardant and plasticizer. This study assessed exposures to BAP or TPP early in life to determine effects on respiration later in life in zebrafish (*Danio rerio*). Whole organismal basal respiration was measured using a swim tunnel. Tissue-specific respiration was then measured for the gonads, liver, heart, and brain of each fish. Fish exposed to BAP exhibited lower total organismal basal respiration compared to controls, while TPP-exposed fish did not have any significant changes. Livers and brains of BAP-exposed fish also had lower mitochondrial respiration, while ovaries were affected in TPP-exposed fish. These findings demonstrate that early-life exposures to BAP and TPP can have profound later-life effects on respiration and mitochondrial function.

EXECUTIVE SUMMARY

The objective of this study was to determine if early-life exposures to benzo(a)pyrene (BAP) or triphenyl phosphate (TPP) caused later-life effects on resting metabolic rate and tissue-specific mitochondrial function in zebrafish (*Danio rerio*). Zebrafish were used as a model organism because they have a high genetic similarity to humans, so the results may potentially be applicable to human health.

BAP is a polycyclic aromatic hydrocarbon that has previously demonstrated toxicity and carcinogenicity in fish. It is an environmental contaminant that can be released through processes involving the combustion of organic matter, including wood-burning, cigarette smoking, and cooking. Due to the ubiquitous nature of PAHs in the environment, it is important to determine if early-life exposures to BAP can cause any significant later-life effects.

TPP is a nonhalogenated organophosphate ester that is commonly used as a flame retardant and plasticizer. It can be found in a variety of consumer products, including furniture, cosmetics, and electronics. It has demonstrated metabolic toxicity, cardiotoxicity, and hepatotoxicity. Due to its frequency of use in products that humans regularly come into contact with, there is a high potential for human exposure. As is the case with BAP, research is needed on how such exposures can affect health over the lifespan.

Fish were exposed embryonically to either BAP, TPP, or vehicle control. BAP exposures were either 0.3 µM or 1.0 µM, while TPP exposures were either 0.1 µM, 0.3 µM, or 1.0 µM. At the ages of 21-24 months old, the fish underwent whole organismal respirometry measurements. Afterwards, the gonads, liver, heart, and brain were removed for analysis of tissue-specific respiration. In both tests, oxygen consumption rate was measured to assess respiration.

The results for BAP showed that whole organismal basal respiration was lower in the exposed groups compared to controls. The liver in exposed fish also showed decreased total basal respiration, mitochondrial basal respiration, and maximal respiratory capacity compared to controls. The results for TPP did not show any significant differences in whole organismal basal respiration. However, there were differences in tissue-specific respiration for the ovaries. The ovaries of the 0.3 µM fish had decreased total basal respiration and mitochondrial basal respiration compared to the controls, although similar differences were not observed in the 0.1 µM or 1.0 µM groups.

The effects of these chemicals on whole organismal respiration and mitochondrial respiration show that early-life exposures to these chemicals can cause significant later-life effects. The decreased whole organismal basal respiration shows that BAP exposures can alter metabolism much later in life, even after the fish lived in a clean environment for almost 2 years. The decreased respiration in the brain, coupled with the results of a previously conducted behavioral study, show that BAP exposure can impact the brain in multiple ways. The results in the liver show that BAP can affect mitochondrial energetics of liver cells which, coupled with its previously demonstrated hepatotoxicity, shows that BAP can cause lasting liver damage. The decreased mitochondrial function in the ovaries caused by TPP could potentially affect reproductive processes. The main takeaway of this study is that even brief early-life exposures of BAP and TPP can have effects on mitochondrial bioenergetics much later in the lifespan.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds identified as environmental pollutants. PAHs can be introduced into the environment through natural causes, such as wood combustion in forest fires, as well as anthropogenic sources, such as vehicle exhaust, cigarette smoke, and coal tar (Abdel-Shafy and Mansour, 2016). PAHs have been shown to exhibit reproductive, developmental, and immunological toxicity in fish and other aquatic organisms (Abdel-Shafy and Mansour, 2016). They are sources of concern for both human and ecological health, as they are harmful to both humans and wildlife species.

PAHs have also been described as mitotoxicants. Mitotoxicants are chemicals that damage mitochondria by disrupting the electron transport chain, disrupting mitochondrial DNA (mtDNA) replication, and generating reactive oxygen species (ROS) (Meyer et al., 2013). Mitochondria are especially susceptible to PAHs because their membranes are high in lipids, making it easier for these hydrophobic compounds to pass through and accumulate (Meyer et al., 2013). Special attention has been paid to mitotoxicants in aquatic ecosystems, where they accumulate and pose risks to the health of fish populations (Jayasundara, 2017).

Benzo(a)pyrene (BAP), a five-ringed PAH and known carcinogen, is a mitotoxicant that can affect mtDNA and nuclear DNA (Jung et al., 2009). Much of BAP's toxicity comes from its metabolites. Its dihydrodiol-epoxide metabolite, (+/-)-7 beta, 8 alpha-dihydroxy-9 alpha, 10 alpha-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, is a toxic metabolite that targets mtDNA and causes cancer (Backer and Weinstein, 1980). BAP and its metabolites have previously been shown to exhibit mitochondrial toxicity in zebrafish larvae (Raftery et al., 2017) .

BAP has also been shown to have tissue-specific toxic effects in fish. In male Atlantic killifish (*Fundulus heteroclitus*), BAP exposure led to decreased weight of the testis as well as

decreased testosterone levels, indicating endocrine disruption (Booc et al., 2014). In female killifish, exposure decreased estradiol production, although ovary weight and egg production were unchanged (Booc et al., 2014). BAP exposure has also caused hepatic tumors and other forms of liver damage, such as an increased number of focal liver alterations (Wills et al., 2010). Zebrafish embryos can develop cardiac defects, decreased heart rate, and altered heart morphology following exposure (Huang et al., 2012).

A study by Gao et al. (2017) found that early-life BAP exposure to zebrafish caused neurodegenerative effects later in life. The neurodegeneration consisted of decreased dopamine and norepinephrine levels, impaired cognition, issues with locomotion, and the loss of dopaminergic neurons (Gao et al., 2017). They also found a significantly elevated number of apoptotic brain cells, which could also contribute to neurodegeneration (Gao et al., 2017).

Triphenyl phosphate (TPP) is an organophosphate ester commonly used as a flame retardant and plasticizer (Reemtsma et al., 2008). TPP has also been used as an ingredient in cosmetics and personal care products such as nail polish, possibly as a replacement for phthalates (Mendelsohn et al., 2016). In laboratory studies, it has shown toxicity in cell cultures of multiple species (i.e., human, dog, monkey) (Mochida et al., 1988). Zebrafish exposed as embryos to TPP developed metabolic disruptions, and DNA damage in livers was observed later in adults (Wang et al., 2016). These authors also reported TPP to cross the blood-brain barrier (Wang et al., 2016). TPP exposure inhibited differentiation of cardiac cells in zebrafish embryos as well as causing heart malformations and reduced heart rates (Qi et al., 2019).

Zebrafish are a laboratory aquarium model fish widely used in environmental and developmental toxicology studies (Bambino and Chu, 2017; Howe et al., 2013). They are easy and inexpensive to maintain in the laboratory (Lawrence et al. 2012). They also have a short

lifespan that allows for relatively rapid studies of development and aging following chemical exposure (McCollum et al. 2011; Ali et al. 2011). Zebrafish are small in size and their anatomy is simple and well-defined, enabling easy dissection and observations of changes in major organs (Dai et al. 2014). Zebrafish also have a high genetic similarity with humans, sharing approximately 70% of their genome (Howe et al., 2013). This means that effects of exposure to certain toxicants in zebrafish may produce similar effects in humans if target genes are shared between the two species.

The objective of this study was to determine later life effects of early life exposure to BAP and TPP. Fish were 21-24 months old during the experiment, with the human age equivalent being middle age, or about 50-60 years old (Gilbert et al., 2014). Organismal and tissue-specific metabolic rates were measured using respirometry in aging zebrafish that had been exposed as embryos.

METHODS

Exposure

Zebrafish embryos were collected from a breeding colony and exposed in the Levin laboratory, Duke University. Briefly, breeder tanks were set up between 4:00-6:00 p.m. and adults allowed to spawn for 1 hour starting at 9:00 a.m. the following morning. Embryos were collected from breeder tanks and placed in Petri dishes containing 30 mL Danieau's medium (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4 \cdot 7H_2O$, 0.18 mM $Ca(NO_3)_2$, 1.5 mM HEPES buffer; pH 7.6).

Zebrafish embryos were exposed to either: control, TPP (0.1 µM, 0.3 µM, or 1.0 µM), or BAP (0.1 µM or 0.3 µM). All groups including control contained 0.1% (1 µL/mL) dimethyl

sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) as a vehicle. Exposures were done in glass petri dishes, with 40 embryos in 40 mL of medium, and there were 4 replicate dishes per group. Exposure began at 5 hours post fertilization (hpf) and ended at 120 hpf. Dishes containing embryos were kept in an incubator at 28°C. Embryos were observed daily for deformities, and any dose that lead to >50% mortality or a visible deformity was excluded. All fish with visible deformities were removed upon identification. After hatch, larvae were washed with fresh Daneau's medium and reared as described below.

Fish Husbandry

Individuals exposed as embryos were grown up in clean conditions in the Levin laboratory. Fish were placed in a recirculating Aquatic Habitats system (AHAB, Pentair Aquatic Eco-systems, Apopka, FL) with a pH of 7.0-8.0, a temperature of 28℃, and a 14:10 light:dark cycle. They were placed on an AHAB system at 6 days of age. For the first 30 days, they were fed powdered food pellets three times per day. Brine shrimp were then phased in and the powdered food pellets were phased out. Once the fish were large enough to eat full size Gemma Micro dry pellets, that became their mid-day meal. The fish were transported to the Di Giulio lab at approximately 20 months old and housed in a recirculating AHAB system under the same conditions.

From the age of 20 months on, the fish were fed twice per day during the week and once per day during the weekend. In the mornings and on weekends, fish were fed *Artemia nauplii* (90% Great Lakes Strain, Pentair Aquatic Eco-Systems) and Zeigler's Adult Zebrafish Complete Diet (Pentair Aquatic Eco-Systems) in the afternoons. All zebrafish procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Whole Animal Oxygen Consumption Rate

A Beamish-style swim tunnel (4000 mL vol; Loligo Systems, Viborg, Denmark) with 170-mL swim tunnel respirometers was used to measure oxygen consumption rates (OCR). Each fish was isolated and fasted for approximately 24 hours prior to OCR measurements. This way, energy expenditure associated with digestion and storage of food (i.e., specific dynamic action (SDS) or thermic effect of food (TEF)) would not affect basal metabolic rate during measurements. Following placement into the swim tunnel, a fish was allowed 1 hour to acclimate under a black tarp to prevent disturbances. Oxygen consumption was measured for 1 hour.

Measurements consisted of recording oxygen concentrations (μmol L^{-1}) using OxyView software (PreSens, Regensburg, Germany). Each measurement contained two values. First, the oxygen value of the tunnel was recorded as the lowest value within a 5 second period. Immediately after, the pump was shut off for 5 minutes. Fish behavior was noted during this period since the level of physical activity could affect the oxygen content of the tunnel. After 5 minutes, the second oxygen value was recorded and the pump turned on. There were intervals of 10 minutes between each set of measurements so the oxygen level could return to normal. A total of 4 sets of measurements were taken for each individual, and fish were then returned to their tanks. For each set of measurements, end values were subtracted from start values to yield OCR (μmol L^{-1}). OCR values were averaged and then divided by the fish's body weight to get the resting metabolic rate (RMR) and then divided by 5 to determine RMR per 5 minutes.

Tissue-specific Oxygen Consumption Rate

A Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA; hereafter referred to as "Seahorse") was used to analyze organ-specific OCR. Dissections were

performed on each fish one day after its whole body respirometry measurements. The fish continued to be isolated and fasted between swim tunnel measurements and dissection. The fish were anaesthetized in an ice water bath for approximately 15 seconds, until loss of equilibrium and movement. Fish were then removed from the bath, weighed (mg wet weight; Mettler Toledo, Columbus, OH, d = 0.01mg/0.1mg), and measured for total length (mm). Cervical dislocation was performed immediately prior to dissection.

On the day of dissection, fresh Ringer's solution was prepared using 8.4 mmol L^{-1} sodium pyruvate, 5.6 mmol L^{-1} $NaHCO_3$, 0.97 mmol L^{-1} HEPES, and 3.2 mmol L^{-1} HEPES sodium salt that was added to a stock of 115 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1.2 mmol L^{-1} $MgCl_2$, 0.64 mmol L^{-1} NaH_2PO_4, and 2.1 mmol L^{-1} $CaCl_2$. The pH was adjusted to 7.0. 525 µl of Ringer's solution was placed into each well of a Seahorse islet capture microplate. For each fish, the brain, liver, heart, and gonads were removed and individually placed into wells, with capture screens placed on top to secure the organs in place. After all of the organs were plated, 250 µl of Ringer's solution was removed and replaced with 250 µl of fresh Ringer's. Plated organs were then placed into the Seahorse.

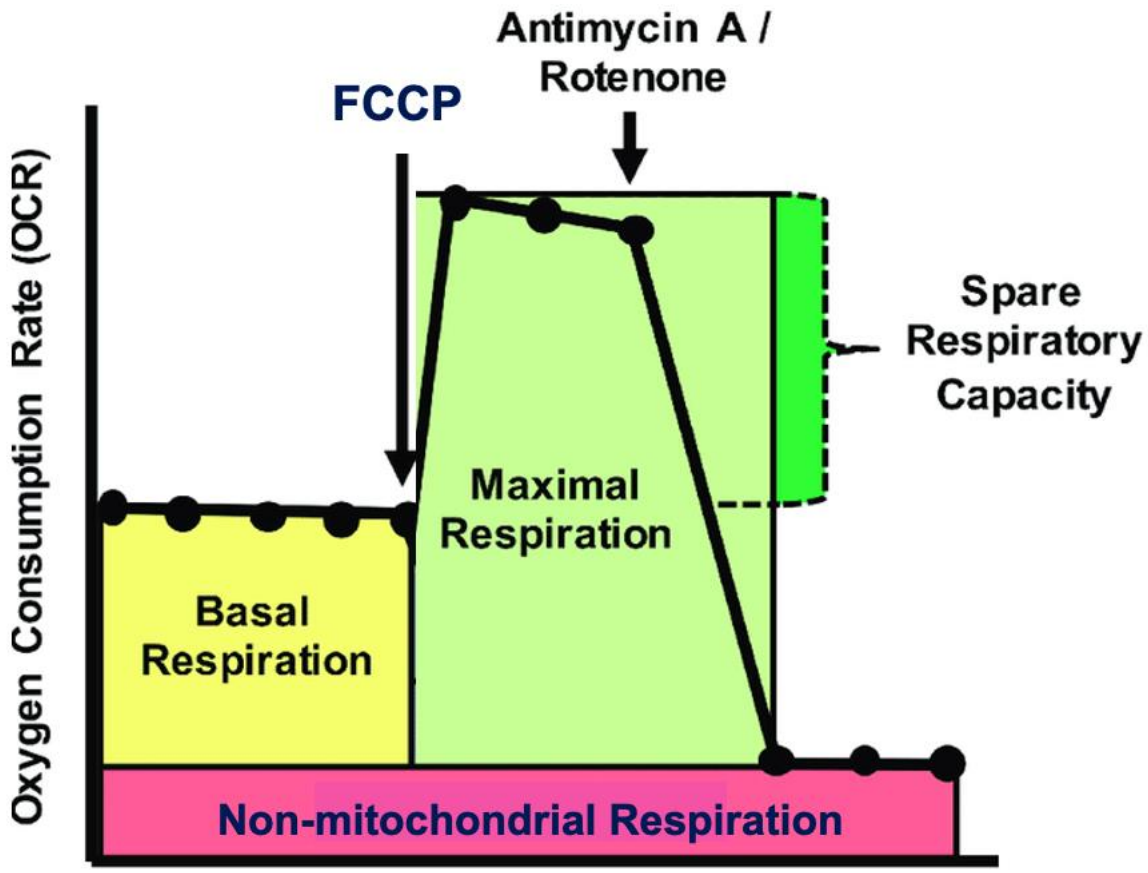

Figure 1: Oxygen consumption rate schematic for tissue-specific respiration (Nicholas et al., 2017).

The oxygen consumption rate schematic shown in Figure 1 illustrates the different measures of oxygen consumption recorded in this study. The first measurement taken is the total basal respiration, or total basal oxygen consumption rate (basal respiration plus non-mitochondrial respiration in Figure 1). Then, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) is injected via the drug ports. FCCP is an uncoupler of oxidative phosphorylation in mitochondria. It simulates a cellular demand for extra energy and causes the mitochondria to act at maximum capacity, giving the value for maximal mitochondrial respiration (Demine et al., 2019). The difference between the maximal mitochondrial respiration and the total basal respiration is known as the spare capacity. The spare capacity of the

mitochondria is the potential of the mitochondria to produce extra energy when the cell has to meet a higher energy demand (Desler et al., 2012).

The second injection consists of a mixture of antimycin A and rotenone. Rotenone is a complex I inhibitor and antimycin A is a complex III inhibitor, so the mixture of the two causes mitochondrial respiration to cease (Wang et al., 2015). With mitochondrial respiration no longer in effect, the remaining oxygen consumption can be attributed to non-mitochondrial respiration. This consists of oxygen consuming processes outside of the mitochondria, including the activity of certain enzymes in the cell (Wang et al., 2018). The non-mitochondrial respiration is subtracted from the total basal respiration in order to calculate the mitochondrial basal respiration.

For this study, eight measurements were taken first to measure basal OCR. Next, 2 µM FCCP in 1% DMSO was injected and then eight measurements of maximal respiration were taken. After that, a mixture of 44.4 µM antimycin A and 44.4 µM rotenone was injected to measure non-mitochondrial respiration for 25 cycles. These measurements were used calculate total basal respiration, total maximal respiration, non-mitochondrial respiration, basal mitochondrial respiration, maximal mitochondrial respiration, and spare capacity. Calculations were normalized by organ weight to obtain the respiration per mg wet weight.

Statistical Analysis

Data were analyzed and graphed using GraphPad Prism version 9.1.0 (GraphPad Software, San Diego, CA). A two-way ANOVA test with a post-hoc Dunnett's test was used to determine significant differences between groups. P-values less than 0.05 were considered statistically significant. Any negative spare capacity values were excluded, as well as the

maximal mitochondrial respiration from that specific tissue. The total basal OCR and mitochondrial basal respiration were not excluded. This is because tissues that had negative spare capacities did not exhibit a response to FCCP. The lack of a response meant that the peak OCR measured for maximal mitochondrial respiration was not accurate, with it sometimes being lower than the total basal OCR. Since the maximal mitochondrial respiration was inaccurate, the spare capacity would also be inaccurate. It is not possible for a spare capacity value to be negative because it represents the extra respiratory potential within the mitochondria. The tissues would still respond to antimycin A and rotenone, so the total basal OCR and mitochondrial basal respiration were still accurate and could be included in the analysis.

RESULTS

Benzo(a)pyrene: Whole Organismal Basal Respiration

There were no significant interactions between treatment and sex in any of the BAP-exposed fish. When both sexes were combined into one group, the 0.3 µM group (n = 17) had significantly lower whole organismal basal respiration than the control (n = 16) ($p = 0.016$, Figure 2). There was also a difference in the 1.0 µM group (n = 18) when compared to the control ($p = 0.051$, Figure 2).

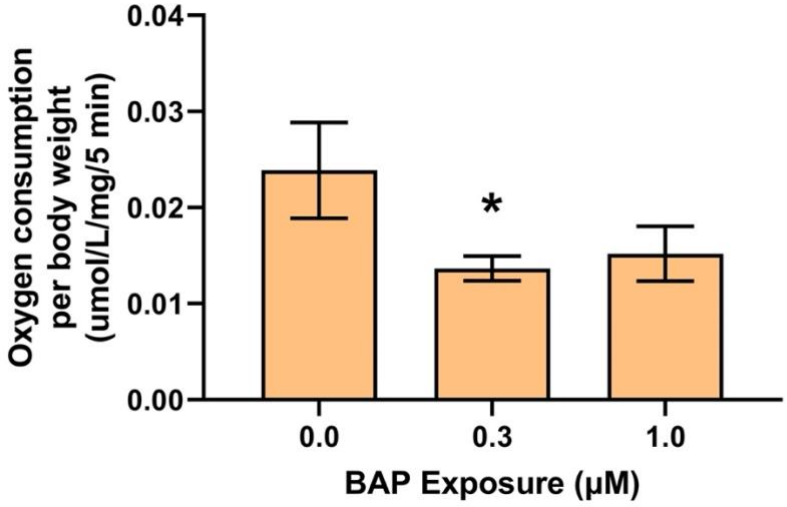

Figure 2: Whole organismal basal respiration for BAP-exposed fish. Asterisk (*) indicates significant difference ($p < 0.05$) between treatment and control (0.0 µM).

Benzo(a)pyrene: Tissue-Specific Respiration

There were no significant interactions between exposure and sex in any of the tissue-specific analyses, so with the exception of the gonads, the sexes were combined into one group for further analyses. There were no significant differences in gonads or hearts of fish exposed to BAP compared to controls. However, there were some significant differences and trends in livers, as seen in Figure 3. The mean total basal OCR for both groups ($n = 17$ for 0.3 µM and $n = 18$ for 1.0 µM) was significantly lower than the control ($n = 15$) ($p = 0.030$ for 0.3 µM and $p = 0.018$ for 1.0 µM; Figure 3A). Mitochondrial basal respiration showed a downward trend for the two treatment groups ($n = 16$ for 0.3 µM and $n = 18$ for 1.0 µM) compared to the control ($n = 14$) (effect of treatment: $p = 0.086$; Figure 3B). Near significant differences in treatment groups ($n = 14$ for 0.3 µM and $n = 13$ for 1.0 µM) compared to control ($n = 12$) were found for maximal mitochondrial capacity (effect of treatment: $p = 0.059$; Figure 3C).

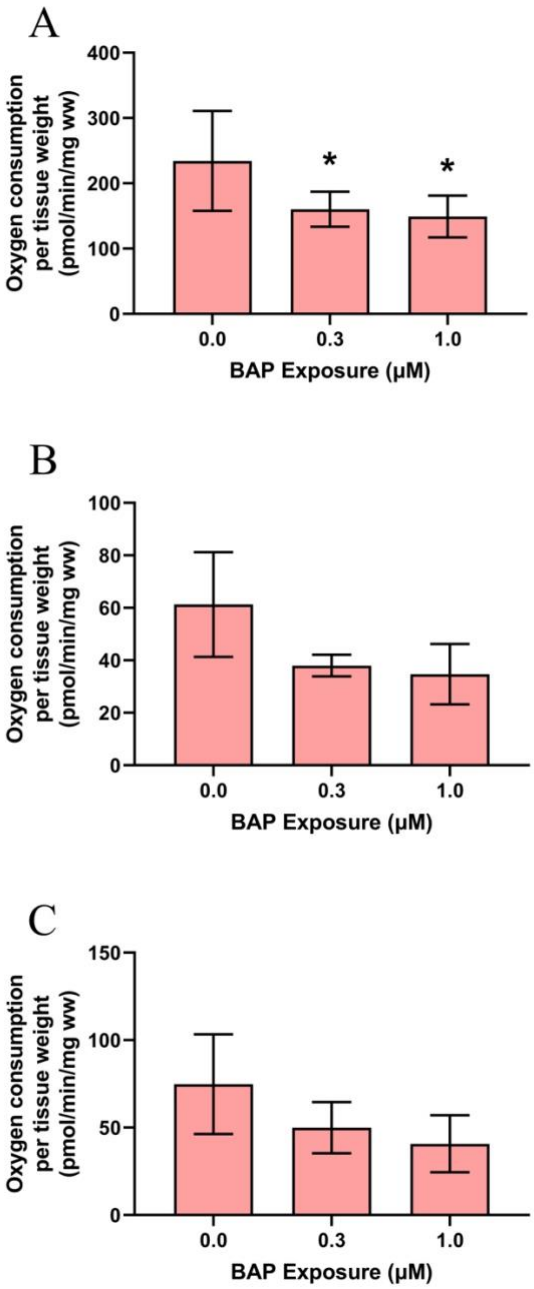

Figure 3: Alterations in livers of BAP exposed fish included A) mean total basal OCR, B) mean mitochondrial basal respiration, and C) mean maximal mitochondrial respiration.

There was also a near significant decrease in mitochondrial basal respiration in the brains of the BAP-exposed fish (n = 17 for 0.3 µM and n = 17 for 1.0 µM) compared to control (n = 16), as shown in Figure 4 (effect of treatment: $p = 0.058$).

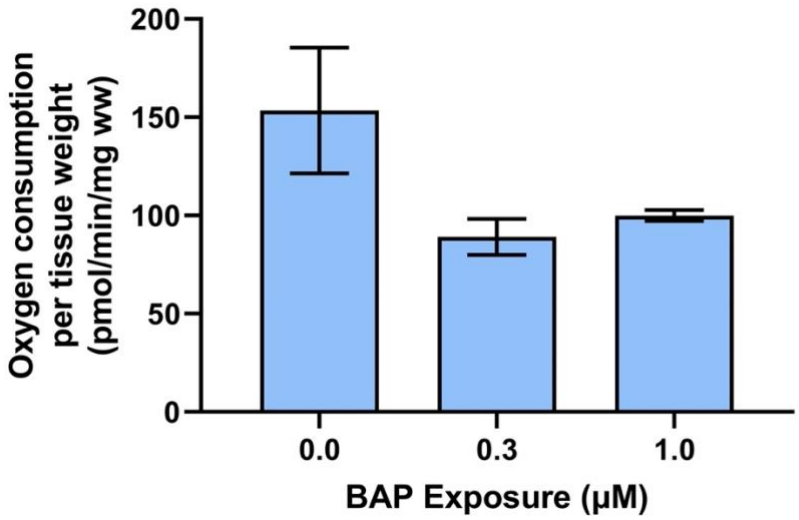

Figure 4: Mitochondrial basal respiration in brains of BAP-exposed fish.

Triphenyl phosphate: Whole Organismal Basal Respiration

There were no significant interactions between treatment and sex for the TPP-exposed fish. There were no significant differences for whole organismal basal respiration in treated fish (n = 16 for 0.1 µM, n = 18 for 0.3 µM, and n = 10 for 1.0 µM) compared to controls (n = 16).

Triphenyl phosphate: Tissue-specific respiration

There were no significant interactions between treatment and sex for the tissues of the TPP-exposed fish, so the sexes were combined within one group for further analyses, excluding the gonads. Fish in the 0.3 µM group (n = 8) had a significantly lower mean total basal OCR in the ovaries than the control (n = 8) ($p = 0.0378$). The 0.3 µM group (n = 8) also had a downward trend for mitochondrial basal respiration in the ovaries compared to control (n = 8) ($p = 0.0768$). There were no differences in either the 0.1 µM (n = 8) or 1.0 µM (n = 5) groups.

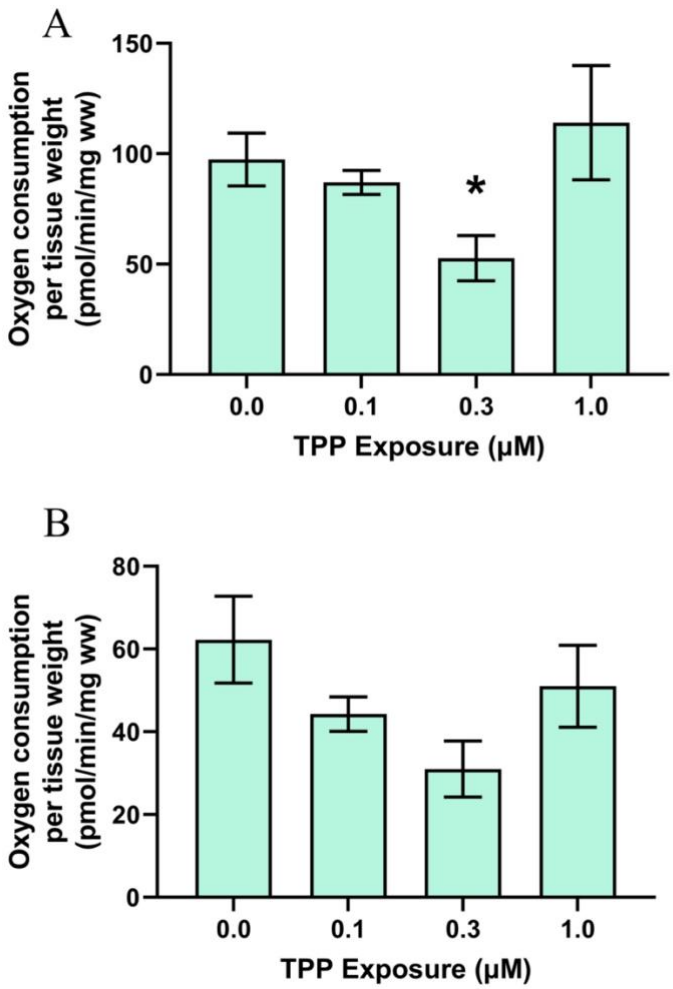

Figure 5: Alterations in ovaries of TPP-exposed fish including A) mean total basal OCR and B) mean mitochondrial basal OCR (b) of the ovaries in TPP-exposed fish. Asterisk (*) indicates significant difference ($p < 0.05$) between treatment and control (0.0 µM).

DISCUSSION

The present study examined the later-life effects of early-life exposure to a PAH and an organophosphate ester. Benzo(a)pyrene and triphenyl phosphate are mitotoxicants known to affect fish during early development (Raftery et al., 2017), (Lee et al., 2019). However, there is a lack of research looking to see if such effects extend into adulthood. The fish in this study

exhibited several changes in respiration two years after the cessation of exposure. The significance of these findings is that almost 2 full years after exposure, during which the fish lived in clean, non-contaminated water, they are still experiencing physiological effects.

Benzo(a)pyrene

Whole Organismal Basal Respiration

The lower levels of whole organismal basal respiration in BAP-exposed fish are indicative of decreased basal metabolism. This is interesting because of the four tissues analyzed, only the liver and brain showed differences in mitochondrial function. This shows that whole organismal basal respiration may not always be directly linked with tissue-specific basal respiration. The cause for this decrease in resting metabolic rate is difficult to pinpoint, but there could be a link to overall mitochondrial function throughout the body. BAP exposure has been shown to cause irreversible mitochondrial DNA damage in Atlantic killifish (Jung et al., 2009). While not necessarily related to respiration, this is evidence of BAP's negative effects on mitochondria in fish.

Embryonic exposure of BAP to zebrafish has also been shown to have transgenerational effects. One study on the matter found that when zebrafish embryos were exposed to BAP (either 5 μM or 10 μM), adult fish in the F2 generation exhibited increased oxygen consumption, indicating an increased resting metabolic rate (Knecht et al., 2017). However, shortly after fertilization (at 24 hpf), the F2 generation exhibited decreased oxygen consumption (Knecht et al., 2017). Even though these F2 fish weren't directly exposed to BAP, this study serves as further evidence that the effects of BAP on respiration can be inconsistent throughout the lifespan and across generations.

Studies have shown that some environmental toxicants can decrease metabolic rate in zebrafish. A study by Mentor et al. (2020) found that exposure to a mixture of endocrine disruptors decreased metabolic rate in larval zebrafish at 2-5 days post-fertilization. One of the explanations from the authors was that a lower metabolic rate could result from altered hormone levels, indicating significant endocrine disruption (Mentor et al., 2020). For example, the leptin pathway regulates oxygen consumption and metabolic rate in both fish and mammals (Dalman et al., 2013). Knockdown of leptin A has been shown to reduce metabolic rate in zebrafish embryos when measured as a decrease in oxygen consumption (Dalman et al., 2013). However, there is a lack of data on whether early-life exposures can cause decreased leptin activity in aging zebrafish. A study on BAP exposure in mice found that exposure did not cause significant differences in leptin levels, although it did cause weight gain without an accompanying increase in food consumption (Irigaray et al., 2006).

Studies have also found effects of BAP on mitochondrial bioenergetics in zebrafish larvae. One study found that exposure to BAP at 100 µg/L and 1,000 µg/L decreased mitochondrial spare capacity (Raftery et al., 2017). In comparison, tissue-specific analyses of BAP-exposed fish in the present study did not find any changes in spare capacity in any of the tissues measured. However, there could be an explanation for a lack of changes in this mitochondrial parameter. The spare capacity is calculated using maximal mitochondrial respiration and basal mitochondrial respiration. If both maximal and basal mitochondrial respiration are affected in a similar manner, that could lead to an insignificant difference in spare capacity, since both components of its calculation are changing with a similar magnitude. Additionally, organs in larvae can function very differently than organs in adults, as they are not

yet fully developed. If BAP affected an organ in larvae, that effect may not persist as the fish ages, due to the organ developing into its mature form.

Tissue-specific respiration: Gonads

BAP exposure did not cause any significant effects on the gonads of either sex. This was an unexpected result considering BAP has been shown to have negative impacts on both the testes and ovaries in fish (Booc et al., 2014). The differences observed in the current study could be due to the effects diminishing with age, or it could be due to differences in gonadal development. Gonadal differentiation in these fish does not occur until 20-30 days after hatching (Metcalfe et al., 2010). On top of that, the gonads don't fully develop until the fish reach sexual maturity around 8-12 weeks of age (Lawrence et al., 2012). This means that adult exposure and embryonic exposure can have vastly different effects. It may be unreasonable to compare the effects on gonads for embryonic and adult exposures, since gonadal precursors may respond to BAP differently than fully mature gonads would.

Unlike other tissues tested in this study, the male and female gonads were not grouped for analysis because they are completely different organs. Because of this, the sample size was only 8 for each gonad. This smaller sample size could have affected the results. BAP has demonstrated toxicity to the gonads through processes other than respiration, so further research on its later-life effects is still warranted.

Tissue-specific respiration: Liver

The BAP-exposed fish showed differences in total basal OCR, mitochondrial basal respiration, and maximal mitochondrial capacity in the liver. BAP has been previously shown to

cause tumors and other types of liver damage in 9 month old Atlantic killifish that were exposed as larvae (Wills et al., 2010). While tumors may not be related to mitochondrial function, this information sets a precedent for BAP exposure causing liver damage in fish.

There has been a wide variety of studies on BAP and its effects on liver function in fish. One study on acute BAP exposure in ovate sole (*Solea ovata*) found that BAP exposure increased the abundance of mitochondria in the liver, along with peroxisomes and lysosomes (Au et al., 1999). The authors speculated that the increase in mitochondria may be due to BAP causing an increased energy demand, requiring more mitochondria in order to meet the cell's metabolic needs (Au et al., 1999). While this is not necessarily damage, it does show that BAP can have direct effects on the mitochondria of liver cells in fish. However, these findings contrast with the findings of the current study, which showed that BAP exposure decreased mitochondrial respiration in fish. This may be due to the fact that the current study was on aging fish after an early-life exposure, as opposed to an acute study measuring short-term effects. It could be that BAP exposure increases energy demand in the period following exposure, potentially in order to maintain survival processes, but then has a completely different effect on mitochondrial bioenergetics in the long-term.

In adult zebrafish, the liver is responsible for many processes including bile production, blood detoxification, plasma protein production, and the storage of lipids, glycogen, amino acids, and iron (Field et al., 2003). The liver is performing many of these physiological functions prior to and just after hatch (Field et al., 2003). It is possible that the detoxification of BAP during the exposure period led to permanent mitochondrial damage that was still evident in old age.

The male and female livers have different functions in fish. For example, the livers of males have a gene-bias towards carbohydrate metabolism, protein transport, and coagulation,

while the livers of females have a gene-bias towards lipid transport and estrogen pathways (Zheng et al., 2013). Approximately 70% of the genes in the liver are expressed at different levels between the sexes (Zheng et al., 2013). Because of the differences in liver function across sex, it was expected that the male livers would react to BAP differently than the female livers. However, no significant differences were found. This suggests that the components of the liver that BAP is affecting are probably those that are shared between the sexes, as opposed to those that are specific to the liver of one sex. As mitochondrial respiration is a complex, multi-step process, there is a variety of components that BAP exposure could have disrupted, making it difficult to pinpoint the exact mechanism. One possible pathway is through altering the mitochondrial matrix. BAP has been shown to decrease the pH of the mitochondrial matrix, which could disrupt the proton gradient that is essential to oxidative phosphorylation (Hardonnière et al., 2016).

In contrast to the findings of this study, BAP has previously been shown to increase mitochondrial respiration in liver cells from rats. The study consisted of an acute exposure to 40 mg/kg body weight of BAP using young female rats, and they found that the exposure caused increased oxidative phosphorylation in liver cells (Salazar et al., 2004). While this shows a precedent for BAP exposures causing increased mitochondrial function in the liver, there are several explanations as to why this is not reflected in the findings of the current study. One is that the current study measured BAP's later-life effects on aging fish. While it is certainly possible that short-term BAP exposure could cause increased mitochondrial function in the liver, it is just as possible that over the course of the lifespan, the impacts could shift in the direction of inhibiting mitochondrial function. While BAP may cause an initial surge of mitochondrial

activity, it may leave lasting physiological effects that can lead to impaired mitochondrial function as an organism ages.

Tissue-specific respiration: Heart

BAP did not cause significant differences in the mitochondrial bioenergetics of the heart, despite being known to have modes of cardiac toxicity. In zebrafish embryos, BAP has caused altered cardiac morphology and reduced heart rate (Huang et al., 2012). The lack of findings in this study could just be due to the effects fading with age. While there were no differences observed in this experiment, the topic does warrant further study, as there may still be mitochondrial interactions with BAP that can potentially influence cardiac health.

Tissue-specific respiration: Brain

Fish exposed to BAP also had decreased mitochondrial basal respiration in the brain, suggesting there may be changes in locomotion, cognition, or behavior. Early-life BAP exposure at concentrations ranging from 0.5 nM to 50 nM has been shown to cause later-life neurodegenerative disease in one year old zebrafish (Gao et al., 2017). Effects of this exposure included decreases in locomotion, cognition, and levels of norepinephrine and dopamine. Those fish also exhibited higher rates of brain cell death (Gao et al., 2017). It is possible that decreased mitochondrial respiration could be connected with some of these symptoms, but additional study will be needed to find linkages. The main takeaway from this finding is that if the mitochondria in the brain are generating less energy, there may not be sufficient energy for ideal brain function, which might lead to negative neurological effects.

Connection to Behavioral Study

This experiment was paired with a set of behavioral studies done on these fish in the Levin laboratory when they were 8 and 14 months of age. Tests were run on a variety of parameters including shoaling, startle responses (i.e., tap test), novel tank exploration, and predator avoidance. Significant later-life effects were found for the tap test. The tap test consisted of two phases: a pre-tap period and a post-tap period. The pre-tap period measured how much non-exploratory swimming the fish showed in a confined space, reflecting their level of arousal. The post-tap period measured how active fish were in response to a stressor (i.e., a tap on the tank). For the pre-tap test, the 1.0 µM BAP group showed reduced arousal compared to controls at 8 months of age, but this difference disappeared when the fish reached 14 months of age. Conversely, the post-tap test showed no significant differences at 8 months but, at 14 months, the 1.0 µM BAP group showed elevated activity and a higher startle magnitude compared to the controls (Hawkey, 2021). As previously stated, the current study found that BAP exposure decreased mitochondrial basal respiration in the brain. These findings suggest that BAP exposure may affect the brain in multiple ways, with a possible link between behavioral deficits and decreased mitochondrial function in the brain.

The behavioral findings also suggest that over the course of the lifespan, certain behavioral effects may diminish while new behavioral effects may emerge (Hawkey, 2021). This can make it difficult to characterize the effects of early-life BAP exposure over a lifetime, as health effects may be inconsistent and can vary widely as an organism moves through the aging process.

Implications for Human Health

BAP is commonly recognized as a serious threat to human health. A study on an industrial city in New Jersey found that humans are at risk of BAP exposure through both inhalation and ingestion. BAP was introduced into indoor air by cooking, cigarette smoking, and the use of combustion appliances, all of which resulted in inhalation by humans (Waldman et al., 1991). The population in the study also experienced BAP exposure through ingestion of cooked foods, which was generated through the combustion of organic matter in the food (Waldman et al., 1991). Due to the high risk of exposure to BAP (and other PAHs) in industrial areas, research on the various health effects of BAP is warranted in order to understand the hazards that exposed populations face.

The findings of the current study suggest that BAP can affect mitochondrial energetics of the liver and brain, as well as resting metabolic rate. Mitochondrial respiration is crucial to the proper functioning of the cells that make up the brain and liver, so any impairments to mitochondrial function represent a risk to human health. Resting metabolic rate is also important for energy balance and maintaining a stable weight, so a decrease in resting metabolic rate can disrupt these processes (Connolly et al., 1999). The fact that BAP exposure at such a young age can cause significant effects at old age in zebrafish suggests that BAP exposures may have lasting effects on human health through the lifespan. Human exposure to BAP, especially early in life, should be limited in order to minimize any negative later-life effects.

Triphenyl Phosphate

Whole Organismal Basal Respiration

TPP exposure did not result in any significant changes to organismal basal metabolic rate in zebrafish. However, past studies have shown TPP can influence oxygen consumption rate in zebrafish embryos (Lee et al., 2019). These authors reported exposure to TPP (0.064 µM and 0.64 µM) decreased basal mitochondrial respiration but not maximal respiration (Lee et al., 2019). These concentrations were similar in scale to those in the current study. The differences in the current study could be due to a multitude of factors. It may be possible that TPP did affect respiration in young zebrafish, but the effects faded with age. This would suggest that the bioenergetic effects of TPP exposure could be inconsistent throughout the lifespan.

Tissue-specific respiration: Gonads

TPP did not affect the testes, but there were noteworthy findings for the ovaries. The 0.3 µM group had significantly decreased total basal respiration, as well as a trend toward decreased mitochondrial basal respiration. TPP has been shown to impede ovary development and egg production in Japanese medaka (Li et al., 2019). The exposure conditions were different than the current study, as the Li study exposed the fish to TPP at ecologically relevant concentrations for 100 days right after hatching. This implies that the gonads were exposed as gonadal differentiation was occurring. Since ovary development and egg production were assessed immediately after the 100-day exposure period, that means the fish were approximately 3 months of age at the time of analysis. In contrast, the fish in the present study were assessed at 21-24 months of age. The main takeaway from this comparison is that TPP exposure can have significant effects on the ovaries on recently exposed adult fish, as well as adult fish that have

been living in clean water for almost 2 years. The decreased respiration in the ovaries in the present study could have potential impacts on fertility and mating processes in fish. If the mitochondria have a smaller energy output, that means there may be less energy available for egg formation and other reproductive processes.

There were no observed differences in the 1.0 µM group. This may also be due to the sample size, as the 1.0 µM group only had 5 fish of each sex in each group. Additionally, it is possible that the affected fish already died out over the course of the past 20 months, leaving only the unaffected fish available for use in this sample. The 1.0 µM group was noted to have a higher mortality than the other groups, so that may be a possibility.

Tissue-specific respiration: Liver

There were no significant differences in mitochondrial energetics of livers in fish exposed to TPP. This was unexpected as TPP has been shown to affect mitochondrial structure and function in liver cells. A study on liver cells from mice found that TPP exposure altered mitochondrial structure, decreasing its average branch per network ratio, as well as decreasing basal mitochondrial respiration (Le et al., 2021). In the current study, the damage caused by TPP exposure could have diminished with age. Alternatively, it could be possible that the zebrafish liver cells might not have reacted the same way as the mouse liver cells to TPP.

Tissue-specific respiration: Heart

TPP exposure did not cause any significant differences in the mitochondrial bioenergetics of the heart. Like BAP, TPP is already known to have modes of cardiac toxicity. In zebrafish embryos, TPP caused morphological alterations and reduced heart rate (Qi et al., 2019). Similar

to the results for BAP, the lack of findings in this tissue could just be due to the effects fading with age. This topic does warrant further study, as there may still be mitochondrial interactions with TPP that can potentially influence cardiac health.

Tissue-specific respiration: Brain

TPP exposure did not yield any significant findings for the brain. This is not always the case in other studies. In mice, maximal mitochondrial respiration increased in microglia cells following short-term (18-hour) exposure to 10 µM or 40 µM TPP, while basal respiration did not change (Bowen et al., 2020). However, these authors stated that the effects were minimal and that increase was likely due to a cell survival process, as they had observed higher death rates at the 40 µM TPP dose (Bowen et al., 2020). There is little evidence that early-life TPP exposure would affect mitochondrial bioenergetics in the brain later in life. However, the literature in this area is not expansive and further research on the topic is warranted.

Connections to Behavioral Study

Despite there not being any noteworthy findings for respiration in the brain, the behavioral study for TPP did find some significant differences. During the tests at 2.5 months of age, the 0.1 µM group exhibited significantly higher locomotion, with no differences in the 0.3 µM group. Later, during the tests at 8 months of age, the 0.3 µM group had significantly lower locomotion than the controls, with no differences in the 0.1 µM group. There were no significant differences when the test was repeated at 14 months of age (Hawkey, 2021). In the predator avoidance test at 8 months of age, the 0.3 µM group showed significantly higher avoidance (measured as distance traveled) from a slow-moving dot that simulated a predator interaction.

When the test was conducted again at 14 months of age, there was no longer a significant difference, similar to the results of the locomotion tests (Hawkey, 2021).

These results show that the effects of early-life TPP exposure can persist into adulthood, but may be inconsistent as an organism ages. Since there were no differences in the brain's mitochondrial activity for any of the TPP-exposed fish, this could mean that the neurological impacts of TPP diminished with age. However, it could also mean that TPP affected the brain through processes unrelated to respiration and energy balance. Although there were no significant effects on the mitochondrial bioenergetics of the brain, the findings of the behavioral study show that early-life TPP exposures can affect the brain well into adulthood, although these effects can change in severity over the course of the lifespan.

Implications for Human Health

The effects of TPP on human health have also been investigated, due to its high potential for human exposure. Since it is used as a flame retardant and plasticizer, it can be found in a wide variety of consumer products, such as hydraulic fluids, floor polish, cosmetics, and furniture (van der Veen and de Boer, 2012). One study on nail polish containing TPP found that the urinary metabolites of TPP increased sevenfold after dermal exposure to the nail polish (Mendelsohn et al., 2016). TPP has also been found in couches purchased after 2005, when the polybrominated diphenyl ether flame retardant (pentaBDE) was phased out and replaced with organophosphate flame retardants (Stapleton et al., 2012). Due to its use in household products, there is a high probability of human exposure. The findings in the current study suggested an effect of TPP exposure on respiration in the ovaries. Considering TPP exposure could possibly affect ovary function, and it has shown to easily absorb through the skin, TPP exposure could

present a hazard to women in regards to egg production and fertility. The fact that an early-life exposure to TPP caused significant later-life impacts on respiration in the ovaries shows that human exposure to TPP should be avoided when possible, especially for babies and children.

Limitations

There were several limitations of the current experiment. One major limitation was that the sample sizes for all of the groups were small. Each group usually only had 8 fish of each sex, aside from the TPP 1.0 µM group, which had 5 fish of each sex. This is not a problem for most of the tissues, as the two sexes were combined into one group, giving a sample size of 16. The issue is for the gonads, which were unable to be combined into one group, due to being completely different organs. The sample sizes for the gonad-specific analyses were thus constrained to 8 fish per gonad, which is a relatively small sample size.

The small sample size is due to the time-consuming nature of the respirometry measurements, as well as the high costs of the tissue-specific analyses. In addition, there were some instances where certain organs had to be excluded from part of the analysis due to a lack of response to FCCP. There were also times that certain organs were unable to be removed from the fish intact during dissection. Additional fish were added to the various groups to compensate for these losses, but it was not possible to compensate for every problem.

Another limitation is that the whole organismal basal respiration for each fish was based on a series of 4 measurements, which took place over the course of one hour. While this is an adequate number and backed by the literature, some fish displayed large amounts of variation in oxygen consumption over the course of the measurement period, making it difficult to discern if

these oxygen consumption rates were each fish's natural respiration rates. This limitation could be minimized by taking measurements for a longer period of time.

Many of the tissues tested in this experiment did not show significant differences with controls. This could be due to a lack of effects, or effects diminishing as the fish aged in a clean environment. It could also be due to mortality. It is possible that affected individuals died, leaving only the unaffected survivors in the sample for this study. The 1.0 µM TPP group had higher mortality than the other groups, hence the smaller sample size. There were trends in the data to suggest fish in this group may have also responded in many of the parameters tested. If lower mortality had occurred, such effects may have been observed.

Conclusions and Future Research

The main takeaway of this study is that early-life exposures to BAP and TPP can still affect biological processes later in life. BAP reduced resting metabolic rate, as well as decreasing mitochondrial respiration in the liver and the brain. TPP reduced mitochondrial respiration in the ovaries. Even after living in a clean system for almost 2 years, the exposed fish still exhibited these effects. This shows that environmental exposures can have lasting effects on mitochondrial bioenergetics and metabolism. While this study used zebrafish as a model organism, the results may potentially be applicable to humans. Exposure to BAP and TPP can both have potentially adverse effects on human mitochondrial function, so exposure to these chemicals should be limited. Release of these chemicals into the environment should also be monitored, as they may potentially affect aquatic ecosystem health through decreasing energy availability in the specified tissues in fish. Future research on this topic could focus on determining if these effects are consistent throughout the lifespan, or if they become exacerbated by old age. Studies on

mixtures are also needed, as it is unknown what combinations of compounds and stressors could produce synergistic effects. Ecological studies of the effects of these chemicals on an ecosystem level may also provide insight into the environmental relevance of these exposures.

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