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Mitochondrial response and resilience to anthropogenic chemicals during embryonic development

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ABSTRACT

Mitochondria are integral to maintaining cellular homeostasis. Optimum mitochondrial function is critical during embryonic development, as they play a key role in early signaling cascades and epigenetic programming, in addition to sustaining an adequate energy production. Mitochondria are sensitive targets of environmental toxins, potentially even at levels considered safe under current regulatory limits. Most mitochondrial analyses have focused only on chemical exposure effects in vitro or in isolated mitochondria. However, comparatively little is known about mitochondrial effects of chemical exposure during vertebrate embryogenesis, especially during the recovery phase following a chemical insult. Here, we used the zebrafish (*Danio rerio*), in a 96-well plate system, to examine mitochondrial effects of 24 chemicals including pharmaceuticals, industrial chemicals, and agrochemicals. We used oxygen consumption rate (OCR) during embryogenesis as a proxy for mitochondrial function. Embryonic OCR (eOCR) was measured in clean egg water immediately following 24 h of chemical exposure and subsequently for an additional 8 h. Each chemical, dependent upon the concentration, resulted in a unique eOCR response profile. While some eOCR effects were persistent or recoverable over time, some effects were only detected several hours after being removed from the exposure. Non-monotonic dose response effects as well as mitochondrial hormesis were also detected following exposure to some chemicals. Collectively, our study shows that mitochondrial response to chemicals are highly dynamic and warrant careful consideration when determining mitochondrial toxicity of a given chemical.

1. Introduction

Mitochondria are essential for eukaryotic cellular development and function. In addition to maintaining energy homeostasis, mitochondria are involved in a number of processes including oxidative stress response, reactive oxygen species (ROS) signaling, apoptotic signaling, Ca^{2+} signaling and regulation, and biosynthesis of macromolecules (Bhatti et al., 2017; Vakifahmetoglu-Norberg et al., 2017; Pathak and Trebak, 2018; Spinelli and Haigis, 2018; Pfanner et al., 2019). Importantly, mitochondria play a critical role in viability and survival of developing embryos (Mishra and Chan, 2014). Throughout embryogenesis, aside from ATP synthesis, mitochondria facilitate signaling cascades (Chandel 2014) and are involved in intracellular communication (Nagaraj et al., 2017). Mitochondrial synthesis of molecules such as acetyl CoA from glucose and NAD+ is important in acetylation and potential epigenetic modifications of the developing embryo (Moussaieff 2015). Consequently, developmental perturbations to mitochondria may have significant later-life health impacts (Xia et al., 2014., Lueng et al., 2013).

Mitochondria are highly sensitive to environmental toxin exposure. Mitochondrial function (e.g., oxidative phosphorylation) and mitochondrial structures (e.g., lipid membranes and mitochondrial DNA) are key targets of anthropogenic compounds such as agrochemicals, industrial chemicals, and pharmaceuticals (Meyer et al., 2018). Furthermore, xenobiotic chemicals may affect mitochondrial regulation of nuclear-cytoplasmic processes (e.g., hormonal activity) and signaling (e.g., ROS, apoptotic), as well as alter mitochondrial ATP synthesis to meet the increased energy demand.

Despite growing interest on mitochondrial toxins and their potential role in the etiology of chronic diseases, research is just beginning to emerge on mitochondrial effects of chemical exposure during embryonic development. Furthermore, current studies are typically

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Table 1
Chemicals, manufacturer, primary source of entry into the environment and mechanisms of mitochondrial toxicity (if known), and concentrations used for treatment of 1 hpf zebrafish embryos in egg water.

Chemical	Primary source/mechanisms of toxicity in mitochondria	Chemical concentrations in egg water (μM). *Initial chemical stock solutions were diluted in 100% DMSO. (DMSO concentrations did not exceed 0.1% in egg water.)			
Amiodarone Hydrochloride (Sigma, 19774824)	Pharmaceutical/**uncoupler**	(0.001)	(0.01)	(0.1)	(1.0)
Arsenic (Sigma, S7400)	Naturally occurring, used in industrial applications/**Disrupts MMP**	2 μg/L (0.026)	10 μg/L (0.133)	50 μg/L (0.667)	500 μg/L (6.67)
Azoxystrobin (Sigma, 131860338)	Fungicide/**inhibits complex III**	0.5 μg/L (1.24)	2.0 μg/L (4.96)	3.5 μg/L (8.67)	5.0 μg/L (12.39)
BDE-47 (Sigma, 5436431)	Flame retardant, used in manufacturing	0.01 mg/L (0.0205)	0.1 mg/L (0.205)	1.0 mg/L (2.058)	10 mg/L (20.6)
Benzo(A)pyrene (Sigma, 50328)	Combustion of organic compounds	0.02 μg/L (0.000079)	0.2 μg/L (0.00079)	2.0 μg/L (0.0079)	20 μg/L (0.079)
Ezetimibe (Sigma, 163222331)	Pharmaceutical	(0.05)	(0.5)	(5.0)	(50.0)
FCCP (Sigma, 370865)	**Mitochondrial uncoupler**	(0.0125)	(0.025)	(0.05)	(0.15)
Fenpyroximate (Sigma, 134098616)	Pesticide/**inhibits complex I**	(0.05)	(0.1)	(0.2)	(0.4)
Fenazaquin (Sigma, 120928098)	Pesticide/**inhibits complex I**	0.05 μg/L (0.163)	0.5 μg/L (1.63)	5.0 μg/L (16.32)	20 μg/L (65.27)
Fluoranthene (Sigma, 206440)	Combustion of organic compounds	0.01 μg/L (0.005)	0.1 μg/L (0.029)	1.0 μg/L (0.148)	10 μg/L (0.741)
Naproxen Sodium (Sigma, 26159342)	Pharmaceutical	1.0 μg/L (0.004)	10 μg/L (0.043)	100 μg/L (0.434)	1000 μg/L (4.34)
Oligomycin (Sigma, 75351)	**Mitochondrial ATP synthase inhibitor**	(0.125)	(0.25)	(0.5)	(1)
Oxybenzone (Sigma, 131577)	Key compound in Sunscreen	0.004 mg/L (0.0175)	0.04 mg/L (0.1753)	0.4 mg/L (1.753)	4 mg/L (17.53)
PFOA (Sigma, 335671)	Industry	0.0035 μg/L (0.000008)	0.007 μg/L (0.000017)	0.07 μg/L (0.00017)	0.7 μg/L (0.00169)
PFOS (Sigma, 77283)	Industry/**decreases MMP**	0.0035 μg/L (0.000007)	0.007 μg/L (0.000014)	0.07 μg/L (0.00014)	0.7 μg/L (0.00139)
Pyraclostrobin (Sigma, 75013180)	Fungicide/**inhibits complex III**	0.16 μg/L (0.0004)	0.8 μg/L (0.002)	4 μg/L (0.010)	20 μg/L (0.052)
Pyridaben (Sigma, 96489713)	Insecticide/**inhibits complex I**	0.006 μg/L (0.00003)	0.012 μg/L (0.0002)	0.06 μg/L (0.0008)	0.3 μg/L (0.004)
Rosiglitazone (Sigma, 122320734)	Pharmaceutical	(0.01)	(0.1)	(1)	(10)
Rotenone (Sigma, 557369)	Pesticide/**inhibits complex I**	0.005 μg/L (0.00001)	0.05 μg/L (0.00013)	0.5 μg/L (0.0013)	5.0 μg/L (0.0126)
Sodium azide (Sigma, 26628228)	Industry/**inhibits complex IV**	(13.25)	(62.5)	(625)	(6250)
TDCPP (Sigma, 13674878)	Industry and pesticide	(0.001)	(0.01)	(0.1)	(1)
Toxaphene (Supelco, N13586)	Insecticide	1 mg/L (2.428)	5 mg/L (12.14)	10 mg/L (24.28)	20 mg/L (48.57)
Tributyltin (Sigma, 7486353)	Industry/Moussaieff	(0.0001)	(0.001)	(0.01)	(0.1)
Trifloxystrobin (Sigma, 141517217)	Pesticide	1 μg/L (0.00003)	6 μg/L (0.0003)	30 μg/L (0.0025)	150 μg/L (0.0245)

conducted using cell culture assays and only primarily focus on acute mitochondrial effects (Nadanaciva et al., 2013). Significant limitations in these studies include lack of insight into putative effects of chemical metabolites derived *in vivo* from parent compounds, and persistent long-term effects.

Continuous discovery of persistent contaminants in the environment, such as PFOS (Perfluorooctanesulfonic acid; a perfluoroalkyl substance) in the drinking water (Hu et al., 2016), indicate the limitations in determining health effects of environmental exposures solely through chemical composition analyses. Therefore, effect-directed analyses of environmental samples can provide insights into long-term health consequences of environmental exposures. To this end, mitochondrial toxicity can be a useful end-point and provide broader insights into potential health outcomes of environmental exposures. For example, mitochondrial toxicity of drinking water samples may serve as an important indicator of overall water quality (Roubicek and de Souza-Pinto, 2017). Thus, higher throughput approaches assessing mitochondrial effects in a whole organismal exposure context, particularly during vulnerable life-history stages (e.g., embryogenesis) are highly valuable when screening for persistent cellular effects of an individual chemical and their mixtures.

Using zebrafish *Danio rerio* as a model, we optimized a 96-well plate mitochondrial function assay to determine embryonic mitochondrial response to contaminants. The zebrafish is a prominent developmental toxicology model (Nishimura et al., 2016) and has a rapid development time, in which major organ systems (e.g., the nervous system, cardiovascular system, kidneys, and liver) are beginning to form by 24 h post fertilization (Kimmel et al., 1995). This enables monitoring whole organismal level effects of exposure to chemicals and their metabolites. Additionally, zebrafish pharmacokinetic properties are highly conserved to that of humans and relevant in the context of monitoring human health (MacRae and Peterson, 2015; Garcia et al., 2016). Indeed, several previous studies have demonstrated the use of zebrafish embryos as a model for investigating chemical effects on whole embryo mitochondria (Stackley et al., 2011; Conlin et al., 2018; Sounders et al., 2018).

Expanding on these studies, the goals of the current study are three fold: (i) optimize a 96-well plate based assay to measure mitochondrial function during embryogenesis, (ii) elucidate mitochondrial effects of developmental exposure to chemical contaminants at environmentally relevant concentrations, and (iii) test the hypothesis that mitochondrial effects of chemical contaminant exposure, even at very low-levels, during early cellular development (cleavage, blastulation, gastrulation, and segmentation) will persist through maturation. Here, we measured embryonic oxygen consumption rate (eOCR) as a proxy for mitochondrial function following 24 h treatment with pharmaceuticals, agrochemicals and other industrially utilized chemicals, especially at concentrations found in aquatic systems. We focused on mitochondrial effects immediately following treatment with a chemical contaminant during the first 24 h of development. Subsequently, we also measured eOCR during an 8 h recovery period from the exposure to determine persistent mitochondrial effects of chemical contaminants. Overall, we present a 96-well plate assay to screen for mitochondrial toxins in an organismal developmental context.

2. Methods

2.1. Toxicity studies

2.1.1. Exposure protocol

AB strain zebrafish embryos were collected and incubated at 28.5 °C in egg water (1 embryo/1 mL) until 1 hpf at which time embryos were

screened for viability. They were then moved to treatment solutions (10 embryos per 10 mL) for 24 h at 28.5 °C. Embryos were treated with egg water supplemented with a given chemical as summarized in Table 1. These concentrations were determined via a literature search to determine environmentally relevant concentrations and low dose ranges that were previously determined to be non-lethal in aquatic organisms (see Table S1 for literature review). Each chemical treatment was conducted in triplicate and none of the treatments showed significant effects on survival or embryonic development.

2.1.2. Mitochondrial toxicity analysis

Embryonic oxygen consumption rate (eOCR) was analyzed using XF96^e Extracellular Flux Analyzer (Agilent Technologies, CA) and was optimized following previous studies (Stackley et al., 2011; Sounders et al., 2018). A key distinction from previous zebrafish embryo studies using the flux analyzer is that, in the current study measurements were made per single embryo. Following incubation in treatment solutions for 24 h, a given embryo from a treatment group was rinsed with egg water 3 times (10 mLs each). Subsequently, it was transferred to 150 uL of egg water contained in an individual well in a spheroid microplate (Agilent Technologies, CA). Each embryo was centered in the spheroid chamber in the bottom of the well and air bubbles were removed.

We then conducted two types of assays. First we determined embryo mitochondrial function following 24 h of exposure to amiodarone, BDE-47, ezetimibe, fluoranthene, naproxen sodium, TDCPP, and toxaphene. We measured eOCR under basal conditions, followed by eOCR measurements after injecting mitochondrial inhibitors to each well. To measure maximum respiratory capacity 6 μM FCCP (carbonyl cyanide4-(trifluoromethoxy)phenylhydrazone, Sigma-Aldrich, CAS370-86-5) was used, and 9.4 μM oligomycin (Sigma-Aldrich, 75,351) was used as an ATP synthase inhibitor. A given well was only injected with either FCCP or oligomycin. Subsequently 6.25 mM sodium azide (NaAz) (Sigma-Aldrich, CAS 26628-22-8) was injected into each well to completely inhibit mitochondria. These concentrations were determined based on previous studies (Stackley et al., 2011; Sounders et al., 2018). The protocol consisted of basal (15 cycles), post injection FCCP or oligomycin (15 cycles), and post injection NaAz (20 cycles) with a 2:00 min mix, 2:00 min wait, and 3:00 min measure period per cycle. In contrast to previous analyses (Sounders et al., 2018), FCCP and oligomycin injections were conducted separately to prevent synergistic effects of both chemicals that were detected in preliminary studies.

In our second approach, we expanded our list of chemicals and assessed eOCR (basal mitochondrial respiration) for ~510 min to determine the initial mitochondrial response as well as the recovery response. The time length of 510 min was chosen to avoid any potential hatching of embryos mid-run thus avoiding potential confounding results. This assay consisted of an initial 12 eOCR measurements followed by a 15 min wait period and five more eOCR measurements. This cycle was repeated seven times. Each measurement corresponded with a 2:00 min mix, 2:00 min wait, and 3:00 min measure time.

Upon completion of each run, eOCR per embryo data were averaged per treatment group. Mitochondria toxicity profile data yielded seven different mitochondrial parameters; basal respiration, reserve capacity (RC = FCCP induced eOCR – basal eOCR), ATP-independent respiration (AI = basal eOCR – oligomycin induced eOCR), maximal respiration (FCCP-induced eOCR), ATP-linked respiration (AL-oligomycin induced OCR), mitochondrial respiration (basal eOCR – NaAz induced eOCR), and non-mitochondrial respiration (NaAz induced eOCR). Data are presented as a percent of control. The 510 min long basal eOCR data are visualized as mean eOCR per treatment group (i.e., chemical concentration) over time.

2.1.3. Statistical analysis

eOCR per treatment were reorganized in R (R Core Team, 2019) and imported to GraphPad Prism 8.0 (GraphPad Software, San Diego California USA) for further analysis. To determine statistical significance between control and treatment groups for mean eOCR value for a given parameter (basal, reserve capacity, ATP-independent respiration, maximal respiration, ATP-linked respiration, and mitochondrial respiration) an ANOVA was conducted followed by a Tukey post-hoc test. Significance was determined by a $p\ value < 0.05$. Time-series data for each chemical for a given concentration were analyzed using a non-liner regression to determine the slope (rate of change in eOCR over time) and the area under the curve (total eOCR). Statistical significance between control and treatment groups for the slope and area under the curve were determined based on two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%, without assuming a consistent standard deviation (GraphPad Prism 8.0, GraphPad Software, San Diego California USA). Significance was determined by a $p\ value < 0.001$. To determine eOCR immediately following removal from the exposure solution, the first five data points were extracted separately. Given the majority of the samples showed statistical significance when comparing the slope between the treatment group and the control group, we validated our statistical analysis with an additional analysis based on R statistical platform (R Studio Inc. Boston, MA). Briefly, we applied a non-parametric and parametric multilevel model to compare changes in eOCR. The multilevel model is separated into two sub-models, with the level-1 sub-model focusing on within-embryo change and the level-2 sub-model focusing on between-embryo changes. To be specific, the level 1 sub-model uses the 'time' variable as well as each individual fish's eOCR growth rate. I.e., $\Upsilon_{ij} = [\pi_{0i} + \pi_{1i}\ (\mathrm{TIME}_{ij}\ -1.29)] + [\varepsilon_{ij}]$. The Υ_{ij} represents the eOCR value for fish i at time j, while π_{0i} represents the intercept of the true change trajectory for fish i, and π_{1i} represents the slope. There is 1.29 subtracted from the time variable because this centers the data, so that the model does not extend back past the data's temporal limits. Random error is measured in the equation using ε_{ij}. The unobserved random error in the level-1 sub-model, ε_{ij}, is assumed to have a normal distribution. The level-2 sub-model consists of multiple parts, with one part for each level-1 growth parameter (the intercepts and slopes). The sub-models must also specify predictors, i.e., the treatment groups. The separate equations are $\pi_{0i} = \Upsilon_{00} + \Upsilon_{01}\ (\mathrm{TREAT\ i}) + \zeta_{0i}$ and $\pi_{1i} = \Upsilon_{10} + \Upsilon_{11}\ (\mathrm{TREAT_i}) + \zeta_{1i}$, where π_{0i}, the intercept, and π_{1i}, are outcomes that are associated with predictors, i.e., the treatment. To determine the statistical differences in eOCR among fishes at different chemical contamination concentration level, we also applied the COANOVA model (Analysis of Covariance). Furthermore, the trained multilevel model can be used to predict the changes in eOCR with the new time location and chemical contamination concentration level.

3. Results

3.1. Chemical treatment specific mitochondrial toxicity profiles

Mitochondrial toxicity profiles for a given embryo were generated by injecting known mitochondrial inhibitors and an uncoupler during the eOCR measurements to the well containing an embryo that was previously treated with a chemical for 24 h. Data showed that every chemical that we tested, with the exception of naproxen sodium, had at least some statistically significant effect on embryonic mitochondria. TDCPP (0.1 μM) was found to have the greatest effect on mitochondrial eOCR by significantly increasing basal eOCR, max respiration, and mitochondrial respiration (Fig. 1). Toxaphene (24.28 μM) significantly decreased reserve capacity and increased basal respiration (Fig. 1). BDE-47 (2.06 μM) significantly reduced non-mitochondrial respiration, whereas fluoranthene (0.148 μM) led to an increase in overall eOCR leading to higher ATP linked respiration (Fig. 1). Amiodarone (0.1 μM) and ezetimibe (5.0 μM) treatment resulted in an increase in reserve capacity and mitochondrial respiration respectively.

3.1.1. Recovery response trajectory following chemical treatments

As expected based on results described in Fig. 1, a unique

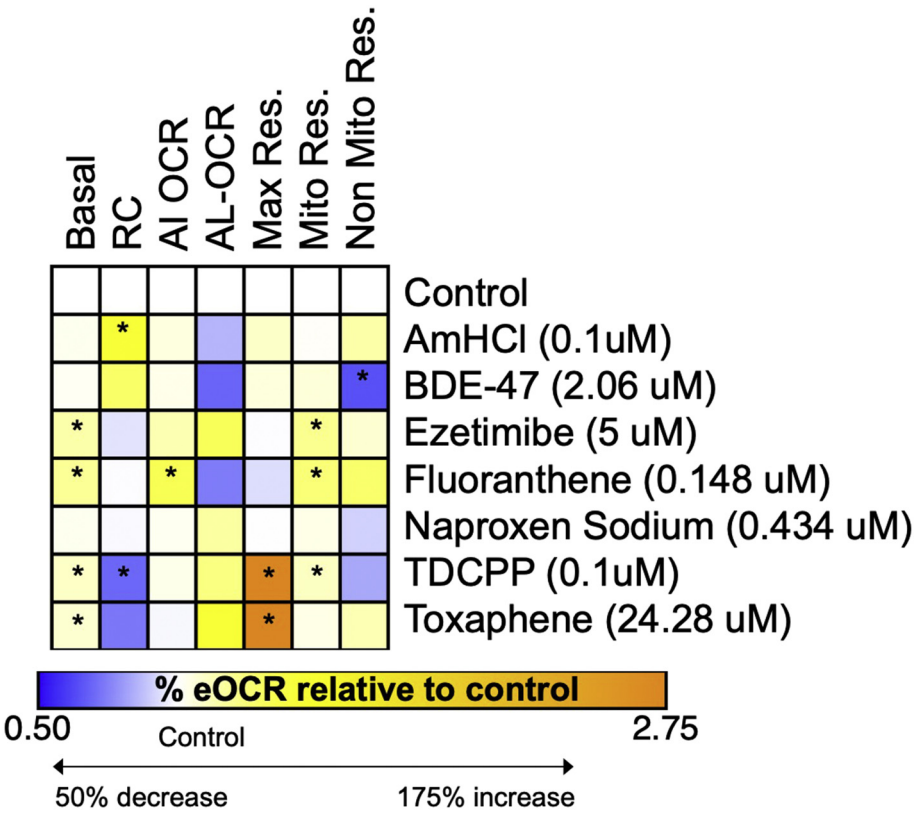

Fig. 1. Heat map representing embryonic oxygen consumption rates (eOCR) in zebrafish treated with AmHCl, BDE-47, ezetimibe, fluoranthene, naproxen sodium, TDCPP, and toxaphene. Embryos were in treatment solution from 1 to 25 hpf. Each square represents percent of control; blue indicates a percent decrease relative to control and yellow indicates a percent increase relative to control. ATP independent respiration (AI OCR) was calculated by subtracting ATP-linked respiration (AL-OCR) from basal values. Mitochondrial reserve capacity (RC) was calculated by subtracting basal value from maximal respiration (Max Res.). Mitochondrial respiration (Mito Res.) was calculated by subtracting non mitochondrial respiration (Non Mito Res.) from basal values. An independent *T*-Test was used to test for significance between treatment and control. Significance is represented by (*), *p-value* $< .05$, $N = 9$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

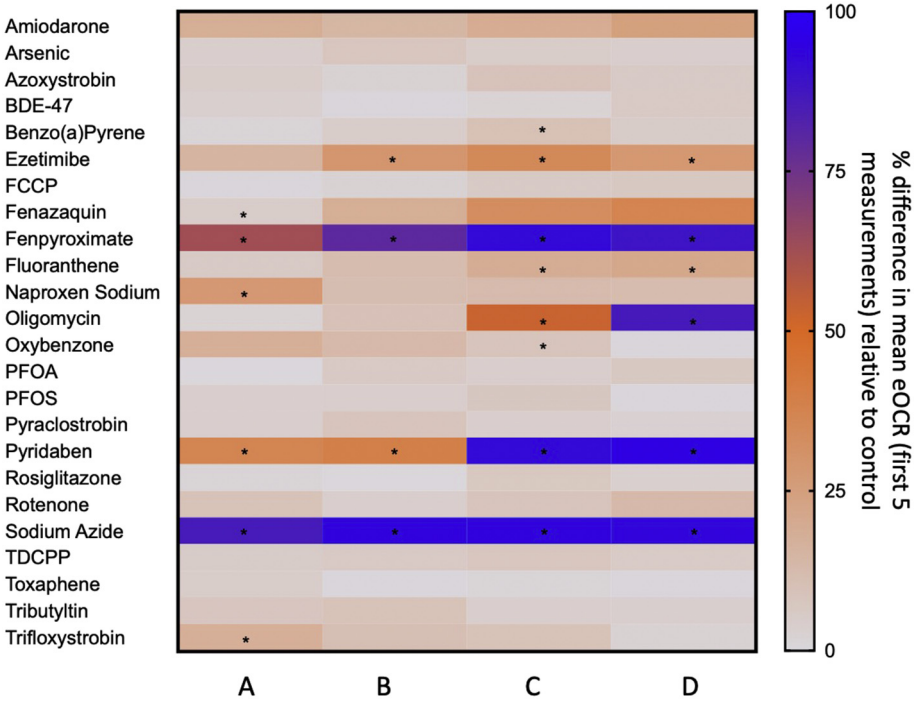

Fig. 2. Heat map representing an initial response (average of first 5 measurements over 24 min) in embryonic oxygen consumption rates (eOCR) in zebrafish treated with a given chemical for 24 h. Embryos were treated at 1 hpf. Each square represents percent difference from control, with white representing 0% change and blue represents maximum percent change. A, B, C, D represents the concentrations used for treatment with each chemical as listed in Table 1. An independent *T*-Test was used to test for significance between treatment and control. Significance is represented by (*), *p-value* $< .05$, $N = 9$ per treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mitochondrial profile was detected per given chemical treatment when considering the recovery trajectory. Eleven chemicals had a significantly altered initial response (average of first 5 measurements, 24 min) relative to controls. Among these, treatments with sodium azide (0.01325, 0.0625, 0.625, 6.25 mM), pyridaben (0.00003, 0.0003, 0.0008, 0.004 μM) and fenpyroximate (0.05, 0.1, 0.2, 0.4 μM) were most toxic to mitochondria, with all 4 concentrations producing a significant increase or decrease in eOCR relative to controls (Fig. 2). This was followed closely by ezetimibe (0.5, 5.0, 50 μM), in which three concentrations significantly increased OCR (Fig. 2).

All chemical treatments at the highest concentration significantly altered basal eOCR over 510 min except for azoxystrobin, ezetimibe, pyraclostrobin, and rosiglitazone (Fig. 3). Treatments with amiodarone, BDE-47, fenpyroximate, PFOA, PFOS, sodium azide, and TDCPP significantly increased or decreased eOCR relative to control under all concentrations (Fig. 3). Following treatment with the lowest

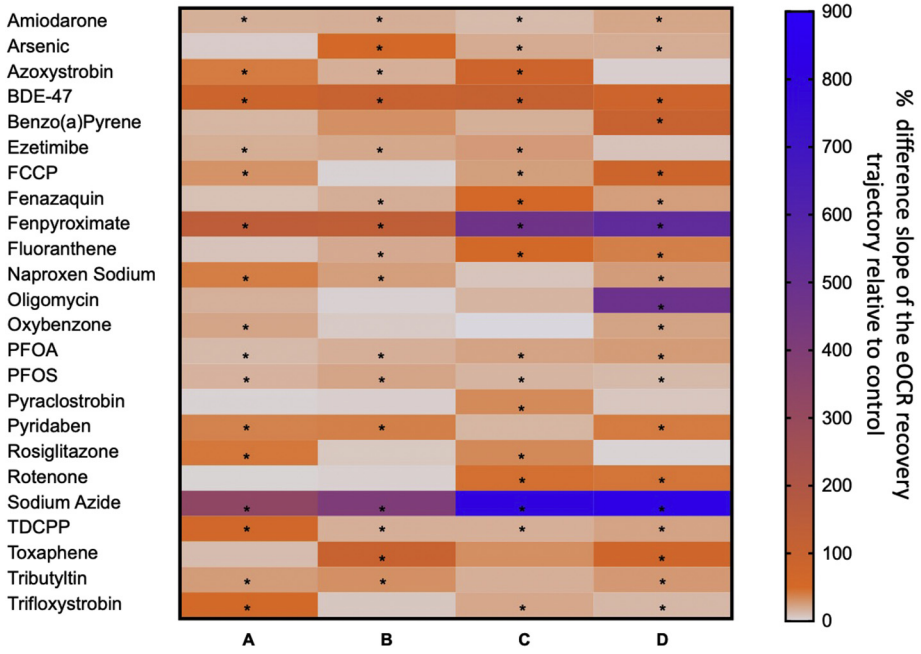

Fig. 3. Heat map representing slope of the recovery trajectory based on a non-linear regression analysis conducted on embryonic oxygen consumption rates (eOCR) over 510 min after zebrafish embryos were removed from a 24 h chemical treatment. Embryos were treated at 1 hpf. Each square represents percent difference from control, with white representing 0% change, and blue represents maximum percent change. A, B, C, D represents the concentrations used for treatment with each chemical as listed in Table 1. An independent T-Test was used to test for significance between slope of the recovery trajectory of treatment and control. Significance is represented by (*), *p-value* < 0.05, $N = 9$ per treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration, 16 of the 24 chemicals also resulted in statistically significant difference in recovery trajectory.

Unsurprisingly, treatments with known potent mitochondrial toxins (e.g., FCCP, oligomycin, rotenone, sodium azide) resulted in significantly decreased basal eOCR at the highest concentrations (Figs. 3, 4A). Sodium azide and oligomycin treatment showed the greatest impact on basal OCR with decreased levels at all concentrations for sodium azide and at 0.5 μM, and 1.0 μM oligomycin (Fig. 4A). Overall recovery responses following embryos treated with FCCP, oligomycin, rotenone, and sodium azide show that changes in eOCR overtime is significantly different compared to the control with a few exceptions—rotenone at $1e^{-5}$ μM and $1.3e^{-4}$ μM and FCCP at 0.025 μM were similar to the controls. Furthermore, oligomycin recovery trajectory (eOCR change overtime) was parallel to that of controls, despite at much lower rates (i.e., the slope of the line was the same for three of the four concentrations tested) (Fig. 4A).

All the other chemicals tested here showed an overall significant effect on eOCR recovery over time (Figs. 3, S2). This was independent of the effects measured immediately following removal from the 24 h exposure (i.e., basal eOCR during the first 5 measurements as shown in Fig. 1). Of all the 24 chemicals tested, 17 of them altered eOCR recovery trajectory in at least 3 of the 4 concentrations tested. Notably, effects of PFOS, PFAS, amiadarone, and fenpyroximate on eOCR trajectory was statistically significant at all four concentrations tested. Benzo-a-pyrene and pyraclostrobin only affected eOCR trajectory at higher concentrations. Conversely, significant effects of azoxystrobin, ezetimibe and rosiglitazone on eOCR trajectory detected at lower treatment concentrations were not evident at the highest concentrations tested. This indicates a non-monotonic dose response.

Non-monotonic dose responses were most prominent following treatment with pesticides fenazaquin and pyridaben. Fenazquin had significantly decreased basal eOCR at 65.27 and 16.32 μM and increased eOCR at 0.163 μM (Fig. 4B). Pyridaben shows decreased eOCR at 0.004 μM and 0.0008 μM and an increase at 0.0002 μM and 0.00003 μM (Fig. 4B).

Amidarone (a pharmaceutical; 0.001, 0.01, 0.1, 1.0 μM) and arsenic (heavy metalloid; 0.133, 0.667, 6.67 μM) both show a mitochondrial hormesis response, in which all treatments of amidarone and the three highest of arsenic result in an increase in basal eOCR relative to control (Fig. 4C). Ezetmibe (a pharmaceutical; 0.5, 5.0, 50 μM) also showed a similar response at the three highest concentrations (Fig. S1A).

4. Discussion

The goal of our study was to develop a 96-well plate assay to elucidate vertebrate developmental mitochondrial effects of exposure to environmental contaminants, some of which are found in drinking water. We were able to deduce chemical impacts on early mitochondrial function via eOCR, given that cellular oxygen is primarily utilized by the mitochondrial electron transport chain during ATP synthesis. Further, by using Xfe96 Eflux technology we were able to subject embryos to specific ETC uncouplers and inhibitors, providing a more in-depth analysis on mitochondrial specific OCR chemical response. With successful implementation of our assay, results show a highly compound and concentration specific mitochondria toxicity profile for a given chemical.

Results also showed statistically significant long-term persistent effects of exposure to environmental contaminants, even at low-levels of exposure, but further investigations are warranted to determine biological mechanisms. Importantly, the analyses of changes in eOCR over time showed a remarkable capacity for mitochondria to recover. For example, direct exposure to potent mitochondrial toxins (e.g., oligomycin and NaAz) reduced cellular eOCR by > 80%; however, when removed from this exposure solution, eOCR increased with time (Fig. 4A). An additional consideration is the relationship between eOCR and oxygen utilized by mitochondrial vs non-mitochondrial processes. As described previously, 80% of the total oxygen consumed during 24 hpf – 48 hpf is utilized by mitochondria. Potential changes in this percent utilization following chemical exposure was not captured in the recovery trajectory assay, but remains an important area of future studies. Further studies focusing on larval (Raftery et al., 2017) and tissue specific (Jayasundara et al., 2015) mitochondrial function are also likely to reveal persistent mitochondrial effects. Nonetheless, the significant differences detected in eOCR recovery trajectory for majority of the treatment groups highlights an important consideration in defining mitochondrial toxicity of a given chemical.

eOCR effects following treatment with potent mitochondrial toxins suggest a concentration dependent, non-monotonic dose response effect. For example, exposure to FCCP, a mitochondrial uncoupler, at concentrations between 0.0125 μM – 0.05 μM induced a very different

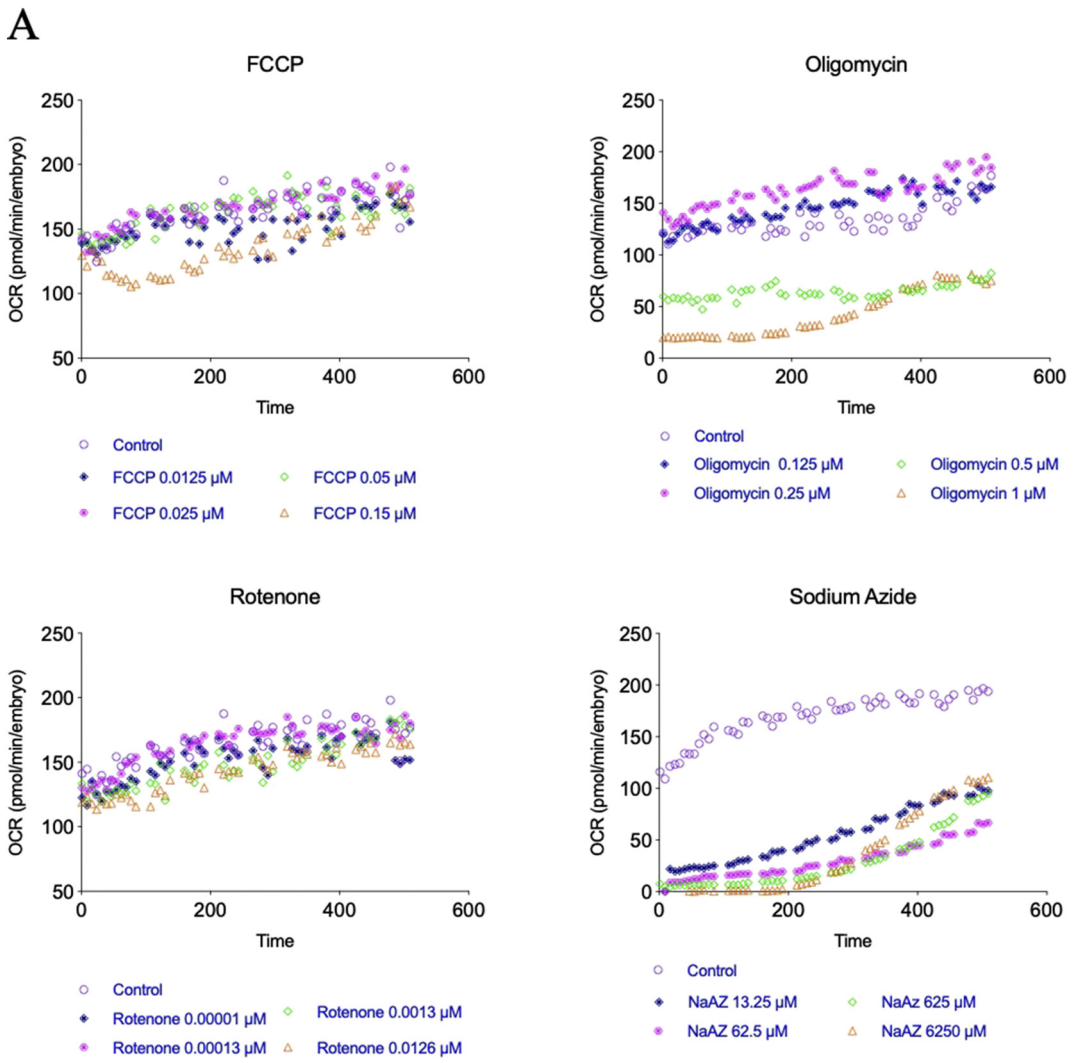

Fig. 4. Several examples of time series data representing basal embryonic oxygen consumption rate (eOCR) over ~ 510 min of 25 hpf zebrafish after 24 h of treatment. Zebrafish were exposed to a gradient of concentrations of a given chemical for 24 h, removed from the exposure solution and eOCR rates were measured for 8 in hours to chemicals. (A) Treatment with mitochondrial inhibition (FCCP, oligomycin, rotenone, sodium azide), (B) chemical treatments depicting non-monotonic dose responses (fenazaquin and pyridaben), and (C) chemicals resulting in mitochondrial hormesis (amidorone, and arsenic), N = 9 per treatment. Statistical significances of the slope and area under the curve for a given treatment is depicted in Fig. 3 and Supplemental Fig. S1.

response compared to 0.5 *μM*. Similarly, eOCR profile following exposure to oligomycin, a mitochondrial ATP synthesis inhibitor, were significantly different between lower (0.125 *μM* and 0.25 *μM*) and higher concentrations (0.5 *μM* and 1 *μM*). Interestingly, despite being highly toxic to mitochondria, FCCP, oligomycin, rotenone and NaAZ, eOCR profiles indicated signs of complete or partial recovery. It is particularly remarkable that eOCR can increase following complete mitochondrial inactivation by NaAz. In fact, partial or complete recovery after 8 h following removal from the exposure solutions were detected with all the chemicals, with the exception of pyridaben and fenpyroximate. However, it is likely that this recovery is temporary. For example, previous studies have shown zebrafish treated with 1 μM oligomycin starting at 5 hpf resulted in increased cardiac edema by 3 dpf (days post fertilization) and decreased survivability by 5dpf (Byrnes et al., 2018). Nonetheless, our data indicate resilience of mitochondria to environmental perturbations during development and the potential capacity to regain homeostasis, at least to some extent. Further studies (e.g., mitochondrial DNA copy number and mitochondrial morphology analyses) are necessary to increase our understanding of mitochondrial recovery following a chemical insult.

Several tested chemicals such as the pesticides fenazaquin and pyridaben, produced non-montotonic eOCR responses in a concentration dependent manner. Treatment with pyridaben at 0.0008 μM resulted in a significant decrease (~14 pmol/min) in eOCR (Fig. 4B). In contrast, pyridaben at 0.004 μM leads to an initial eOCR response similar to 0.0008 μM exposure, but then increases peaking around 6.5 h (just below control values) followed by a decrease. Pyridaben is known to be a mitochondrial complex I inhibitor and has been found to be toxic to rat N27 dopaminergic neuronal cells. At concentrations from 0.5–6 μM pyridaben significantly decreasing OCR after 3 h of exposure (Charli et al., 2016). Here we provide further support that pyridaben affects cellular OCR at very low concentrations in a significant concentration dependent manner.

Treatment with some chemicals led to an increase in eOCR relative to controls, which can potentially be characterized as mitochondrial hormesis. This pattern was seen with arsenic at 0.133 μM and especially at 0.667 μM. Amiodarone also showed a similar response at all the concentrations tested. Data from the mitochondrial toxicity profile analyses (Fig. 1) indicate that this increase in eOCR is coupled to an increase in mitochondrial reserve capacity, suggesting a true mitochondrial hormetic effect. Increases in mitochondrial activity have been reported under low arsenic exposures (Schmeisser et al., 2013). It

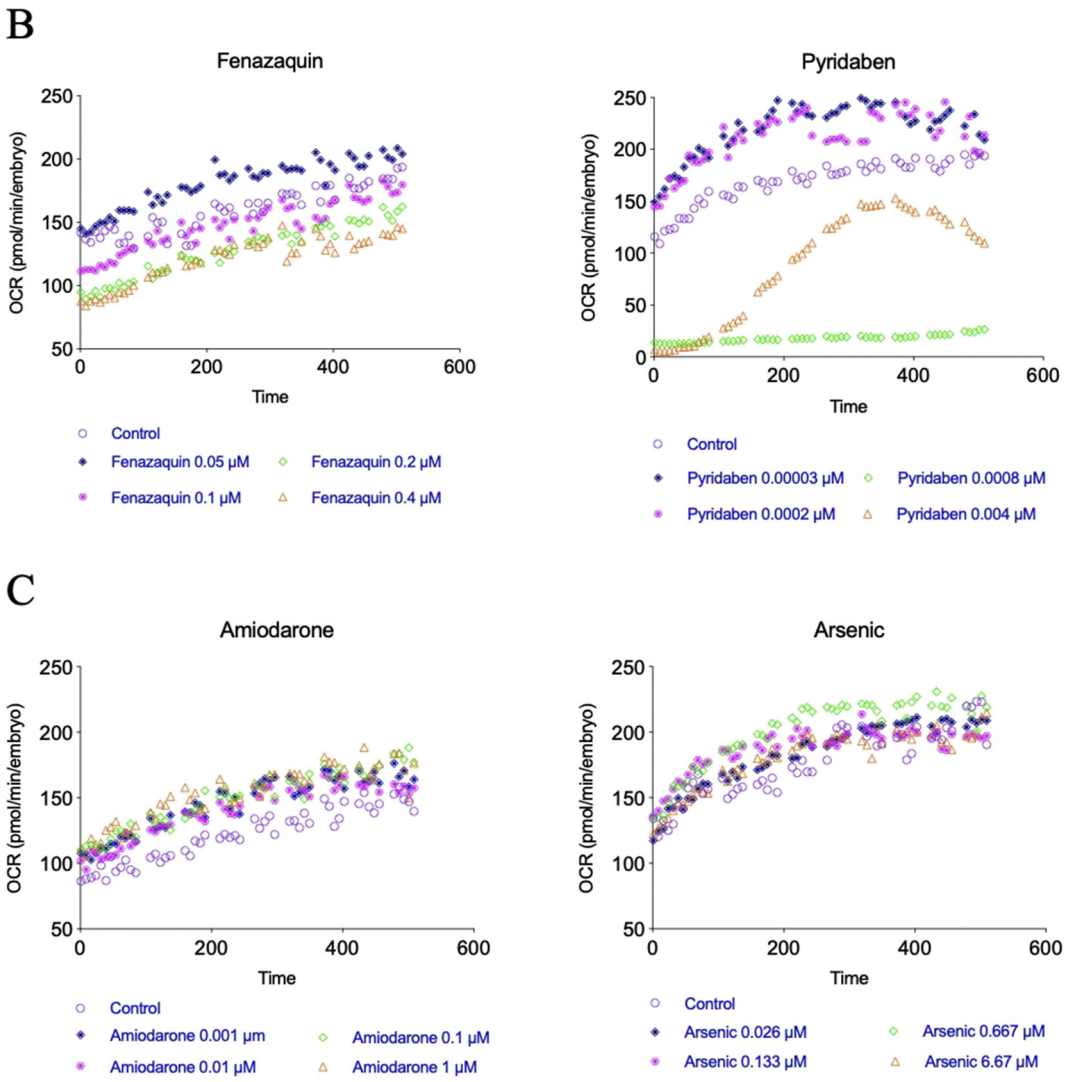

Fig. 4. (*continued*)

is speculated that mitohormesis may be playing a protective role (Meyer et al., 2017) potentially via signal transduction to the nucleus to ultimately regain homeostasis (Merry and Ristow, 2016). When a mitohormesis response is consistently seen after exposure, it would be compelling to explore if there are any later-life fitness consequences, including those that are potentially beneficial.

Given that successful early development of healthy mitochondria is critical, a recovery in eOCR or an apparent insignificant response may still result in later life defects. Previous studies show that exposure to chemicals at the levels examined in our study resulted in long-term physiological effects that may be explained by mitochondrial changes. For example, a 96-h embryonic exposure to low levels of BaP manifested an increase in cellular apoptosis and neurodegeneration in adult stages (Gao et al., 2017). Treatment with pyraclostrobin from 4 hpf to 5 dpf led to reduced body length, decreased OCR, and an increase in ROS related transcripts (KuMar et al., 2020). Studies also show that early chronic exposures to NaAz (Byrnes et al., 2018) result in developmental delays and pyridaben perturbs larval endothelium development (McColluM et al., 2017). Therefore, it is possible that an initial change or significant difference in OCR over time is a key indicator that a chemical is impacting mitochondrial function which may lead to greater developmental stress and later life consequences.

While the eOCR recovery trajectory analyses clearly demonstrated persistent effects on cellular bioenergetics, mitochondrial profile analyses provided further insights into potential mechanisms of action for a given chemical. For example, the increase in OCR with TDCPP at 0.1 µM following 24 h of exposure was coupled to an overall increase in maximum respiratory capacity, inferring an overall increase in total mitochondrial activity. However, given that respiratory capacity was significantly reduced, it appears this increase in maximal respiration is a potential partial compensatory response to the increase in basal eOCR. Toxaphene also followed a similar mitochondrial profile following 24 h of exposure. In contrast, compared to TDCPP, BDE-47 shows a complete opposite effect on mitochondria. Previous studies show that both these chemicals are linked to mitochondrial dysfunction (Byun et al., 2015; Hongmei et al., 2017; Hanyan et al., 2018).

Collectively, our study presents a tool to assess mitochondrial effects of chemical exposure during development using the zebrafish model. We show that this is a highly efficient screening tool that can be used to determine mitochondrial toxicity of pharmaceuticals, industrial by-products, and agrochemicals *in ovo*. Additionally, our eOCR recovery analyses indicate the importance of considering long-term effects of exposure to chemical contaminants, even at low-levels. Future studies may be directed at exploring mechanisms (e.g., examining mitochondrial morphology) and later-developmental consequences of exposure to chemical contaminants toxic to mitochondria. It is possible that positive or negative selection of mitochondria during embryonic development may contribute to altered mitochondrial phenotype following chemical exposure (Otten et al., 2018). This is based on the hypothesis that mitochondria bottleneck during development, where

the total mitochondrial copy number reduces from ~1.9 × 10^6 to ~150 (Otten et al., 2016), before rapidly increasing, is a sensitive window for selection. In addition to mechanisms, with the existing data, further insights may be gained from predictive statistical analyses such as association analyses, an unsupervised machine learning method, that will help to identify the effective combination of various chemical treatments with different concentration levels. In addition, the eOCR data are a typical time-series data, and several types of machine learning methods for time-series forecasting, e.g., random forest, gradient boosting regressor, time delay neural networks, can be applied to discover common toxicity response patterns and underlying mechanisms. Overall, the current study provides intriguing insights into persistent mitochondrial effects of chemical exposure and develop a framework for mechanistic and statistical inferences to better understand mitochondrial toxins.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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