Gill filament permeabilization: A novel approach to assess mitochondrial function in sheepshead minnows (Cyprinodon variegatus) following anthraquinone exposure

DOI:
10.1016/j.cbpc.2019.108699

Document Version
Accepted author manuscript

Published in:
Comparative Biochemistry and Physiology. Part C: Comparative Pharmacology

Comparative Biochemistry and Physiology, Part C

Gill Filament Permeabilization: A Novel Approach to Assess Mitochondrial Function in Sheepshead Minnows (Cyprinodon variegatus) following Anthraquinone Exposure

--Manuscript Draft--

Manuscript Number:	
Article Type:	Method article
Section/Category:	Crude oil toxicity
Keywords:	Oroboros Oxygraph; Mitochondrial Respiration; ROS Production; Proton Leak; photo-modified PAH; Gill Filaments
Corresponding Author:	Dane Crossley II University of North Texas Denton, TX United States
First Author:	Amanda Reynolds Kirby
Order of Authors:	Amanda Reynolds Kirby
	Gina L.J. Galli
	Janna L Crossley
	Lauren E. Sweet
	Dane A. Crossley II
	Aaron P. Roberts
Abstract:	Anthracene is a highly toxic polycyclic aromatic hydrocarbon (PAH), and its toxicity is increased 8-fold after compounding exposure to UV radiation. Exposure to either the parent or photo-modified compound has been shown to cause increases in reactive oxygen species (ROS) production and lipid peroxidation. Since the majority of ROS production occurs within mitochondria, we investigated simultaneous mitochondrial respiration and ROS production in the gills of sheepshead minnows (Cyprinodon variegatus) acutely (48h) exposed to anthraquinone (40 µg l-1). Anthraquinone exposure caused a 25% increase in oxidative phosphorylation with electrons donated to Complex I (OXPHOSCI) and a 33% increase in Leak respiration with oligomycin (Leak-OmyCI). ROS production was slightly increased (33.3%) in Leak state with oligomyocin respiring on Complex I substrates (Leak-OmyCI) after anthraquinone exposure, but this value remained unchanged in all other respiratory states. When ROS production was normalized to mitochondrial oxygen consumption, we found that ROS production was decreased in all respiratory states, but most noticeably in the Leak state. We speculate that differences in the antioxidant defense system may have played a role in decreased ROS production. Overall, in this paper we present a novel technique to measure mitochondrial function in the gill filaments of teleost fish exposed to xenobiotic molecules, and we show anthraquinone exposure alters aspects of oxidative phosphorylation and ROS production.
Suggested Reviewers:	Gigi Lau OsloMet - storbyuniversitetet gigi.lau@ibv.uio.no Mitochondrial Researcher
	Georgina Cox University of Guelph cox@zoology.ubc.ca Environmental Toxicology and Fish
	Rasphal Dhillon The University of British Columbia dhillon@zoology.ubc.ca Mitochondrial Researcher
	Andrew Esbaugh University of Texas at Austin

	a.esbaugh@austin.utexas.edu Previous mitochondrial work and environmental toxicology on fish
	Elena Fabbri Universita di Bologna elena.fabbri@unibo.it Researcher in environmental toxicology
	Kevin Brix The University of British Columbia kevinbrix@icloud.com Environmental Toxicology
Opposed Reviewers:	

Dear Dr Grosell,

Please find accompanying this letter our submission of the manuscript entitled: Gill Filament Permeabilization: A Novel Approach to Assess Mitochondrial Function in Sheepshead Minnows (*Cyprinodon variegatus*) following Anthraquinone Exposure for consideration for publication. In this manuscript we discuss our work in developing a novel technique to measure mitochondrial respiration in the gill filaments of a fish exposed to a single PAH. We have made every effort to follow the formatting requirements of CBP C.

Thank you for your consideration.

Dr Dane A Crossley II

Highlights

- Novel Gill Filament Preparation to Measure Mitochondrial Respiration and ROS Production
- Acute Anthraquinone Exposure Increased Respiration and ROS production in OXPHOS and Leak states
- Observed Results may be Evidence of Mounting Antioxidant Defense Response to Xenobiotic Molecues

Gill Filament Permeabilization: A Novel Approach to Assess Mitochondrial Function in Sheepshead Minnows (*Cyprinodon variegatus)* following Anthraquinone Exposure

A. Reynolds Kirby[1], Gina Galli[2], Janna Crossley[1], Lauren E. Sweet[3] Dane A. Crossley II[1*], Aaron P. Roberts[3]

[1] Developmental and Integrative Biology Division, Department of Biological Sciences, University of North Texas, Denton, Texas

[2] Faculty of Medical and Human Sciences, University of Manchester, Manchester, United Kingdom

[3] Advanced Environmental Research Institute, Department of Biological Sciences, University of North Texas, Denton, Texas

*Corresponding Author: Dane A. Crossley II; email: dane.crossley@unt.edu

Abstract

Anthracene is a highly toxic polycyclic aromatic hydrocarbon (PAH), and its toxicity is increased 8-fold after compounding exposure to UV radiation. Exposure to either the parent or photo-modified compound has been shown to cause increases in reactive oxygen species (ROS) production and lipid peroxidation. Since the majority of ROS production occurs within mitochondria, we investigated simultaneous mitochondrial respiration and ROS production in the gills of sheepshead minnows (*Cyprinodon variegatus*) acutely (48h) exposed to anthraquinone (40 μg l^{-1}). Anthraquinone exposure caused a 25% increase in oxidative phosphorylation with electrons donated to Complex I ($OXPHOS_{CI}$) and a 33% increase in Leak respiration with oligomycin ($Leak\text{-}Omy_{CI}$). ROS production was slightly increased (33.3%) in Leak state with oligomyocin respiring on Complex I substrates ($Leak\text{-}Omy_{CI}$) after anthraquinone exposure, but this value remained unchanged in all other respiratory states. When ROS production was normalized to mitochondrial oxygen consumption, we found that ROS production was decreased in all respiratory states, but most noticeably in the Leak state. We speculate that differences in the antioxidant defense system may have played a role in decreased ROS production. Overall, in this paper we present a novel technique to measure mitochondrial function in the gill filaments of teleost fish exposed to xenobiotic molecules, and we show anthraquinone exposure alters aspects of oxidative phosphorylation and ROS production.

Keywords: Oroboros Oxygraph; Mitochondrial Respiration; ROS Production; Proton Leak; photo-modified PAH; Gill Filaments

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a carcinogenic compound found in smoke, tar, and crude oil. PAHs are released into the atmosphere by the burning and distilling of coal ore (Dipple, 1985), and into aquatic environments through the harvesting and transportation of crude oil (Pasparakis et al., 2019). Some PAHs contain photodynamic properties that are responsive to wavelengths in the UVA spectrum (315-400 nm) and can magnify toxicity in aquatic systems, resulting in photo-enhanced toxicity (Roberts et al., 2017). Photodynamic PAHs are excited by UVA light (380 nm), resulting in the formation of excited singlet and triplet state molecules. These molecules are highly reactive with their surrounding media and generate photo-modified PAHs (byproducts), as well as reactive oxygen species (ROS), which enhances overall toxicity leading to oxidative stress (Arfsten et al., 1996; Mallakin et al., 1999). The photo-modified PAHs are typically oxidized, resulting in higher water solubility and bioavailability to aquatic organisms than the parent PAHs. These molecules are highly reactive in tissues and attach to membrane lipids, which compromise cellular and organelle membrane integrity (Sikkema et al., 1994).

Without the influence of UV, anthracene is a highly toxic PAH with an LC_{50} of approximately 16.6 µg $l^{-1,}$ which is enhanced eight-fold (LC_{50} of 1.94 µg l^{-1}) after co-UV exposure (Weinstein and Polk, 2001). Bluegill sunfish (*Lepomis macrochirus*) exposed to 12 µg l^{-1} of anthracene resulted in 100% mortality within nine hours of direct UV exposure (Bowling et al., 1983). The photo modification of anthracene (as a result of co-UV exposure) forms over 20 toxic byproducts (including anthraquinone), and generally the photoproducts are more toxic than the parent compounds (Mallakin et al., 1999). In the case of anthracene, 50% growth inhibition in aquatic duckweed (*Lemna gibba*) was achieved at a concentration of 1.0 mg l^{-1}, while the

same growth inhibition was achieved in lower concentrations (0.1 mg l^{-1}) (Huang et al., 1995). Photodynamic PAHs (anthracene) absorb UV radiation resulting in an excited energy state and oxidized photoproducts (anthraquinone). The energy released as the excited compound returns to the ground state and can be transferred directly to cellular O_2 or membrane lipids (Roberts et al., 2017). This process of photosensitization has been proposed as the mechanism of toxicity (for PAHs and their photoproducts) through the production of reactive oxygen species (ROS), resulting in oxidative damage to tissues and membranes Mallakin et al., 1999; Roberts et al., 2017; Weinstein and Polk, 2001). In fact, markers of oxidative stress (increased lipid peroxidation and ROS production) were found in bluegill sunfish liver microsomes after 60 minutes of exposure to anthracene (3.015 µg ml^{-1}) and UV light (Choi and Oris, 2000).

Considering that ROS production occurs in the mitochondrial complexes, we wanted to measure the effects of anthraquinone on mitochondrial function (oxygen consumption and coupling efficiency) while simultaneously measuring ROS production in sheepshead minnow gill filaments. We chose the gill filament as a target tissue because PAHs easily flux across the gill epithelium and are rapidly dispersed into the bloodstream (Ramachandran et al., 2006). In the past, oxidative phosphorylation of the gills has been measured in purified isolated mitochondria. However, this preparation requires large tissue samples, which is not possible in small fish species, such as the sheepshead minnow. To this end, we have developed a novel, whole permeabilized gill filament preparation to assess mitochondrial function and simultaneous ROS production in sheepshead minnows (*Cyprinodon variegatus)* following acute (48h) anthraquinone exposure. Permeabilization of the tissue removes the influence of the antioxidant defense systems and cascade signaling and gives us access to the electron transport chain for pharmacological manipulations (Pesta and Gnaiger, 2012). Here we show that the permeabilized

gill preparation is suitable for mitochondrial measurements and can be successfully utilized for toxicological investigations.

Materials and Methods

Animals

Adult, mix-sexed Sheepshead Minnows (*Cyprinodon variegatus)* were purchased from Aquatic Biosystems (Fort Collins, CO) and overnighted to the University of North Texas. Upon arrival, minnows were housed in 100 l tanks filled with 25 ppt saltwater maintained at 26 °C. Saltwater was mixed in the lab by combining deionized facility water with Instant Ocean® Sea Salt (United Pet Group, Blacksburg, VA). Minnows were kept on a 10:14 hour light:dark photoperiod and fed *Artemia* sp. flake food ad libitum every other day, except 24h prior to experimentation. Water quality (pH [6.8-7.5], ammonia [< 5 ppm], nitrite [< 1.0 ppm], nitrate [< 40 ppm]) parameters were monitored daily.

Acute (48h) Anthraquinone Exposure

Adult minnows (two per replicate) were moved to 20l exposure tanks filled with 25 ppt saltwater maintained at 26°C. One tank contained anthraquinone (40 µg l^{-1}) dissolved in DMSO, and the other tank contained an equal volume of DMSO (dimethyl sulfoxide) to serve as a vehicle control. After 24h, minnows were transferred to new aquaria that were filled with 25 ppt saltwater and a dose of either anthraquinone (40 µg l^{-1}) or DMSO (equal volume). A 48h static renewal exposure is a fairly standard procedure when assessing compound toxicity and is used as a precaution of biodegradation and volatilization (Weinstein and Polk, 2001). The concentration of anthraquinone used in this study is within environmentally relevant levels (less than 10 µg ml^{-1}) for anthracene and related byproducts. Temperature and oxygen saturation were monitored throughout experimentation using a handheld YSI dissolved oxygen probe (YSI, Inc, Yellow

Springs, OH). Water parameters (pH, ammonia, nitrate, and nitrate) were monitored throughout the exposure period. After 48h, the trial was terminated and minnows were euthanized using a lethal dose of buffered MS-222 (100mg l^{-1} MS-222 and 200 mg l^{-1} $NaHCO_3$). Gill baskets were removed from both sides of the fish and immediately processed according to the procedures below.

Permeabilized Gill Filament Preparation

Gill arches were placed in ice-cold BIOPS (pH 7.1) containing [2.8 mM CaK_2EGTA (pH 7.0), 7.2 mM K_2EGTA (pH 7.0), 5.8 mM Na_2ATP, 6.6 mM $MgCl_2 \cdot 6H_2O$, 20 mM Taurine, 15 mM Na_2 Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, and 50 mM 2-(N-morpholino) ethanesulfonic acid] (Galli et al., 2016). Under a Lecia EZ4 dissecting scope (Lecia Microsystems Inc., Buffalo Grove, IL) with a dark background, mucous and blood clots were removed from the gill arches (figure 1). Gill arches were incubated at 4°C in BIOPS with saponin (50 µg ml^{-1}) on an orbital shaker (Cole-Parmer, Vernon Hills, IL) for 12 minutes at speed two. Arches were triple washed (to remove excess lipids) for 10 minutes in 4°C MIR05 respiration medium (pH 7.1) containing [0.5 mM EGTA, 3.0 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-2-(N-morpholino) ethanesulfonic acid, 20 mM Taurine, 10mM KH_2PO_4, 20mM HEPES, 110 mM D-Sucrose, 1% BSA] (Galli et al., 2016). Gill filaments were cut from the arches and wet weight was recorded. Gill filaments (wet weight 2 mg) were added to each chamber of an Oroboros Oxygraph 2K HR respirometer (Oroboros Instruments, Innsbruck, Austria) containing MIR05 respiration solution maintained at 30°C. The chambers were hyper-oxygenated and oxygen concentration was maintained between 200-400 nm ml^{-1} during each trial. Mitochondrial function analyses were conducted in two parts: 1) measurement of mitochondrial respiration with simultaneous ROS production and 2) a cytochrome c test with measurement of $OXPHOS_{CIV}$.

This was done because cytochrome c and TMPD with ascorbate (complex IV substrates) are strong redox substances that consume H_2O_2 which interferes with the H_2O_2 calibration and overall sensitivity of the method (Makrecka-Kuka et al., 2015).

The Oroboros was outfitted with O2K-fluorescence LED probes (Oroboros Instruments, Innsbruck, Austria) to detect ROS flux within the Oxygraph chambers. The following protocol measuring simultaneous ROS production and mitochondrial respiration is modified from previously published studies (Krumschnabel et al., 2015; Suski et al., 2012). Amplex™ UltraRed (10 µM), horseradish peroxidase (1 U/ml), and superoxide dismutase (10 U/ml) were injected into each chamber to induce oxidation of Amplex™ UltraRed to red-fluorescent resorufin in the presence of H_2O_2. The fluorescent signal was calibrated by stepwise injections of 400 µm H_2O_2 (final concentration; 0-2 mM).

Protocol 1: Respiratory states and ROS production

Respiratory substrates malate (2 mM) and pyruvate (5 mM) were injected into the chambers and served as a measurement for LEAK respiration without adenylates while respiring on Complex I substrates (LEAK-LN_{CI}; table 1). Succinate (10 mM) was added to the chamber to measure LEAK respiration with Complex I and II substrates without adenylates (LEAK-LN_{CI+CII}; table 1). $OXPHOS_{CI+CII}$ (table 1) was induced by adding saturating levels of ADP (5 mM). Oligomycin (2 µg ml^{-1}) was then added to the chambers to block the ATP synthase and measure LEAK state with Oligomycin (LEAK-$Omy_{CI+CII;}$ table 1). Rotenone (0.5 µM) was titrated into the chambers to inhibit Complex I of the ETS and measure LEAK-Omy_{CII} (table 1). Lastly, Antimycin A (2.5 µM) was added to the chambers to measure residual oxygen consumption (ROX) or chamber respiration not attributed to mitochondrial respiration (table 1).

Protocol II: Cytochrome C test and OXPHOSC_{IV} Respiration

Malate (2mM), and pyruvate (5mM) were added to provide carriers for the citric acid cycle. Saturating concentrations of ADP (5mM) were injected into the chambers to initiate $OXPHOS_{CI,}$ and succinate (10 mM) was added to provide substrates for complex II activation ($OXPHOS_{CI+CII}$; table 1). Cytochrome c (10 µM) was added to assess mitochondrial quality and to determine inner mitochondrial membrane integrity after permeabilized gill filament preparation (table 1). $OXPHOS_{CIV}$ was stimulated through tetramethyl-p-phenylene-diamine (TMPD; 0.5mM) and ascorbate (2 mM) injections (table 1).

Calculations and Statistical Analysis

Trials were excluded from the data analysis when respiration rate increased above 15% after cytochrome c injection and when respiratory control ratios (RCR) were lower than 6, which indicated a damaged preparation (Brand, 2011). All fluxes were corrected for background O_2 consumption by subtracting the respiration rate after gills were added but before mitochondrial substrates and inhibitors were added. Respiration rate and ROS production were normalized to the wet weight of the gill filaments and expressed as pmol sec^{-1} mg wet wt^{-1}. The respiratory control ratio (RCR) was calculated by dividing $OXPHOS_{CI+CII}$ respiration rate by $LEAK_{LNCI+CII}$ in protocol I. All statistical tests were performed using Statistica Version 13.3. A repeated-measures ANOVA with a Tukey HSD test was used to make comparisons in mitochondrial respiration and ROS production between treatments. A student's t-test was used to check for difference at $OXPHOS_{CIV}$ and RCRs between control and exposed minnows.

Results and Discussion

To date, the effects of individual PAH exposure on intact mitochondrial function are relatively unknown. Few studies have highlighted the effects of crude oil exposure on

mitochondrial function, which provided the framework for our investigation. We have developed a new preparation for measuring mitochondrial function in whole gill preparations from small fish. Furthermore, we have shown exposure to anthraquinone effects aspects of mitochondrial electron transport chain function and ROS production.

Development of a permeabilized whole-gill mitochondrial preparation

Permeabilized mitochondrial preparations allow detailed characterization of electron transport chain function in their normal intracellular position, thus preserving essential interactions with the cytoskeleton and other organelles. We adapted protocols for skeletal muscle preparations to develop a permeabilized whole-gill preparation (Fig. 1A). Our RCR values (control: 3.88 ± 0.49 and exposed: 4.42 ± 0.42) from permeabilized gill filaments are consistent with published values in teleost cardiac and skeletal muscle (Fig. 1B; Chung et al., 2017; Guderley and Johnston, 1996; Iftikar and Hickey, 2013). Furthermore, there were negligible effects of Cytochrome C, which suggests the inner mitochondrial membrane was intact. Taken together, these results suggest the fibers were of excellent quality with no overt signs of dysfunction from the permeabilization process (Brand, 2011). Therefore, we consider this preparation is appropriate for studying mitochondrial function in whole-gills, which is particularly useful for studies in small fish.

Effects of anthraquinone on Electron Transport Chain Function and ROS production

There was a tendency for anthraquinone exposure to cause an increase in oxygen consumption across all respiratory states. This effect was particularly pronounced, and statistically significant, when Complex I and II substrates were combined in both the OXPHOS and Leak-Omy states (Fig. 1B). The fact that both Leak and OXPHOS increased with anthraquinone exposure meant that RCR's ratios were similar between exposed minnows and

controls (3.88 ± 0.49 and 4.42 ± 0.44 respectively), suggesting that mitochondrial efficiency remained unchanged. These results suggest anthraquinone exposure increases mitochondrial oxidative capacity in minnows. Given that ATP demand is increased during toxic exposure (e.g., recruitment of ATP-dependent ion channels), a greater mitochondrial capacity may represent a compensatory mechanism to match ATP supply to demand during environmental stress.

Anthraquinone exposure led to a 30% increase in ROS production during Leak-Omy$_{CI+CII}$ respiratory state (Fig. 1D). However, a different pattern emerged when ROS production was normalized to mitochondrial oxygen consumption. For all leak respiratory states (Leak-Ln$_{CI}$, Leak-Omy$_{CI+CII}$, Leak-Omy$_{CII}$), ROS production was reduced by 20 to 40% in mitochondria from minnows exposed to anthraquinone (Fig 1E). Therefore, after differences in oxygen consumption are taken into account, our results suggest ROS production is lower in minnows exposed to anthraquinone, compared to controls. While we did not address the mechanism underlying the differences in ROS production, it is possible that minnows exposed to anthraquinone may mount a protective response by actively suppressing pathways involved in ROS production, or upregulating the antioxidant defense system (Regoli et al., 2011). Support for the latter hypothesis comes from studies in liver mitochondria of goldfish (*Carassius auratus*), which showed levels of superoxide dismutase and catalase were elevated in exposed to phenanthrene exposure (a PAH that is abundant in aquatic systems) (Yin et al., 2007). Nevertheless, it is possible that the exposure length or concentration was not long or high enough to overwhelmed the antioxidant defense system, leading to an increase in ROS production, similar to previous studies (Yin et al., 2007). Further research is necessary to resolve these speculations, which could include measurements of antioxidants and markers of oxidative damage (lipid peroxidation).

In contrast to our study, Xu and colleagues (2017) found that larval red drum (*Sciaenops ocellatus)* exposed to crude oil concentrations of 4.8 µg l^{-1} ΣPAH_{50} at 24,h, 48h, and 72h had differential transcription profiles that were consistent with changes in mitochondrial function, mitochondrial transmembrane potential, oxidative stress response pathways, and cell death signaling (Xu et al., 2017). In adult red drum, mitochondrial function and performance was assessed following 24h exposure at concentrations of 29.6 ± 7.4 µg l^{-1} and 64.5 ± 8.9 µg l^{-1} ΣPAH_{50} and they found no change in Leak respiration, maximal ETS, or coupling controls ratios (markers of mitochondrial dysfunction) after 24h of crude oil exposure (Johansen and Esbaugh, 2019). Mitochondrial function has also been measured in adult Mahi-mahi (*Coryphaena hippurus*) that were exposed to crude oil (9.6 ± 1.7 µg l^{-1} ΣPAH_{50}) for 24h and the authors determined that while $OXPHOS_{CI}$ and $OXPHOS_{CII}$ respiration was decreased, classic markers of mitochondrial dysfunction were not observed (no change in leak respiration, maximal ETS, or coupling control ratios) (Kirby et al., 2019). It should be mentioned that the crude oil concentrations used in the 24h exposures are environmentally relevant to concentrations that occurred during the Deepwater Horizon Oil Spill (Wade et al., 2011) and the lowest doses were previously shown to affect swim performance and aerobic scope in adult fish (Johansen and Esbaugh, 2017; Mager et al., 2014). Taken together, these studies and ours suggest the effects of crude oil and their constituents on fish mitochondrial function are variable and unpredictable. Clearly, more research needs to be conducted in other areas of mitochondrial bioenergetics to determine the mechanisms of toxicity that crude oil and individual PAH have on teleost mitochondrial function.

Acknowledgments

This research was made possible by a grant from The Gulf of Mexico Research Initiative. Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (doi: 10.7266/N71Z4304).

Tables and Figure Legends

Table 1: The action and subsequent mitochondrial states as a result of substrates and inhibitors used in Protocol I and II.

	Action	State	Electron Entry	Abbreviation
PROTOCOL I				
Malate (2 mM) pyruvate (5 mM)	Citric acid cycle and glycolysis intermediates. Substrates for Complex I-linked respiration	LEAK respiration without adenylates (LN)	Complex I	LEAK-LN_{CI}
Succinate (10mM)	Citric acid cycle intermediate. Substrate for Complex II-linked respiration	LEAK respiration without adenylates (LN)	Complex I & II (convergent electron entries into the Q-junction)	LEAK-LN_{CI+CII}
Saturating ADP (5mM)	Substrate for ATP synthase	OXPHOS respiration	Complex I & II	$OXPHOS_{CI}$
Oligomycin (2 µg ml^{-1})	Inhibitor of the F_1F_oATPase (Complex V)	LEAK state induced with oligomycin	Complex I & II	LEAK-Omy_{CI+CII}
Rotenone (0.5 µM)	Inhibitor of Complex I	LEAK state induced with oligomycin	Complex II	LEAK-Omy_{CII}
Antimycin A (2.5 µM)	Inhibitor of Complex III	Residual oxygen consumption	N/A	ROX
Respiratory Control Ratio	Indicates the degree of coupling OXHPOS and ATP synthase	$OXPHOS_{CI-CIV}$ / Leak-LN_{CI+CII}	N/A	RCR
PROTOCOL II				
Malate (2 mM) pyruvate (5 mM)	Citric acid cycle and glycolysis intermediates, respectively. Substrates for Complex I-linked respiration	LEAK respiration without adenylates (LN)	Complex I	LEAK-LN_{CI}
ADP (5mM)	Substrate for ATP synthase	OXPHOS respiration	Complex I & II	$OXPHOS_{CI}$
Succinate (10mM)	Citric acid cycle intermediate. Substrate for Complex II-linked respiration	LEAK respiration without adenylates (LN)	Complex I & II	$OXPHOS_{CI+CII}$
Cytochrome C (10 µM)	Component of the electron transport chain	N/A	N/A	N/A
Ascorbate (2mM)	Antioxidant to prevent autoxidation of TMPD (see next step)	N/A	N/A	N/A
Tetramethyl-p-phenylene-diamine (TMPD; 0.5mM)	Electron donor to Complex IV	OXPHOS respiration	Complex IV	$OXPHOS_{CIV}$

Figure 1: Gill arches (A) extracted from a Sheepshead Minnow (*Cyprinodon variegatus)* and viewed under a Lecia EZ4 dissecting scope prior to permeabilization. Protocol one measurements of mitochondrial respiration (B) and simultaneous ROS production (D) after 48h of exposure in control tanks (open bars) or in anthraquinone (40 $\mu g\ l^{-1}$) dosed tanks (closed bars). Measurements from protocol two on OXPHOS respiration (C). ROS production normalized for mitochondrial respiration rate (E). Data are represented as means ± s.e. Single asterisk denotes significant differences between treatments ($p < 0.05$).

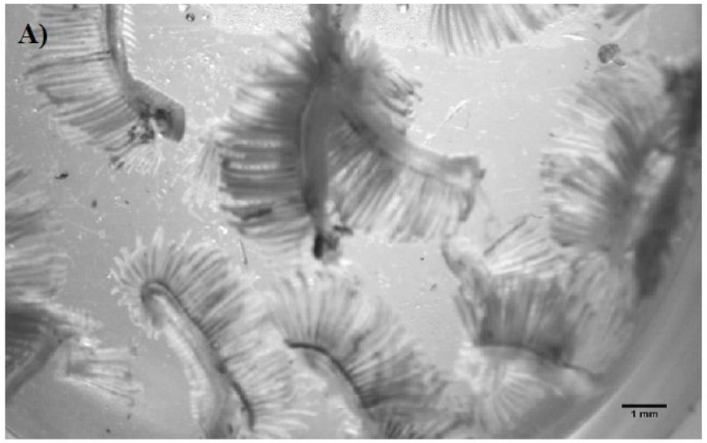
References

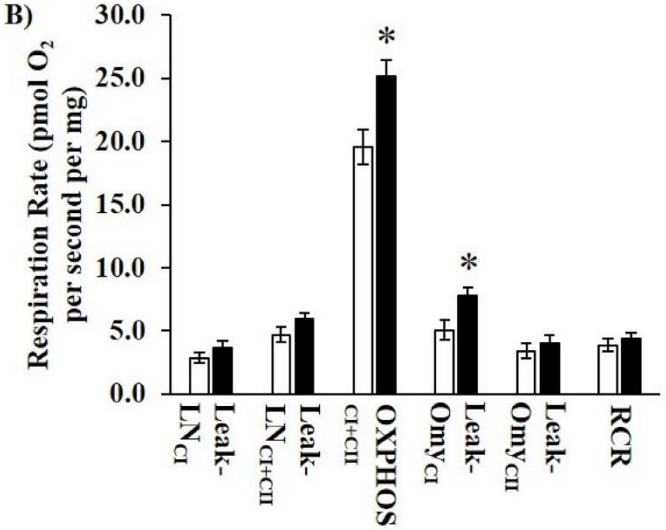
1. Arfsten, D. P., Schaeffer, D. J., & Mulveny, D. C. (1996). The Effects of Near Ultraviolet Radiation on the Toxic Effects of Polycyclic Aromatic Hydrocarbons in Animals and Plants: A Review. *Ecotoxicology and Environmental Safety, 33*(1), 1-24. doi:http://dx.doi.org/10.1006/eesa.1996.0001
2. Brand, M.D. and Nicholls, D. G., 2011. Assessing mitochondrial dysfunction in cells. *Biochemical Journal*, *435*(Pt 2), 297-312. doi:10.1042/BJ20110162
3. Bowling, J., Leversee, G., Landrum, P., & Giesy, J. (1983). Acute mortality of anthracene-contaminated fish exposed to sunlight. *Aquatic Toxicology, 3*(1), 79-90.
4. Choi, J., and Oris, J. T., 2000. Evidence of oxidative stress in bluegill sunfish (Lepomis macrochirus) liver microsomes simultaneously exposed to solar ultraviolet radiation and anthracene. *Environmental Toxicology and Chemistry*, *19*(7), 1795-1799. doi:10.1002/etc.5620190713
5. Chung, D.J., Bryant, H.J., Schulte, P.M., 2017. Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (Fundulus heteroclitus). The Journal of Experimental Biology 220, 1459-1471.
6. Dipple, A.1985). Polycyclic Aromatic Hydrocarbon Carcinogenesis. *283*, 1-17. doi:10.1021/bk-1985-0283.ch001
7. Galli, G.L.J., Crossley, J., Elsey, R.M., Dzialowski, E.M., Shiels, H.A., Crossley, D.A., 2016. Developmental plasticity of mitochondrial function in American alligators, Alligator mississippiensis. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 311, R1164-R1172.
8. Guderley, H., Johnston, I., 1996. Plasticity of fish muscle mitochondria with thermal acclimation. Journal of Experimental Biology 199, 1311-1317.
9. Huang, X.D., Dixon, D.G., Greenberg, B.M., 1995. Increased Polycyclic Aromatic Hydrocarbon Toxicity Following Their Photomodification in Natural Sunlight: Impacts on the Duckweed Lemna gibba L. G-3. Ecotoxicology and Environmental Safety 32, 194-200.

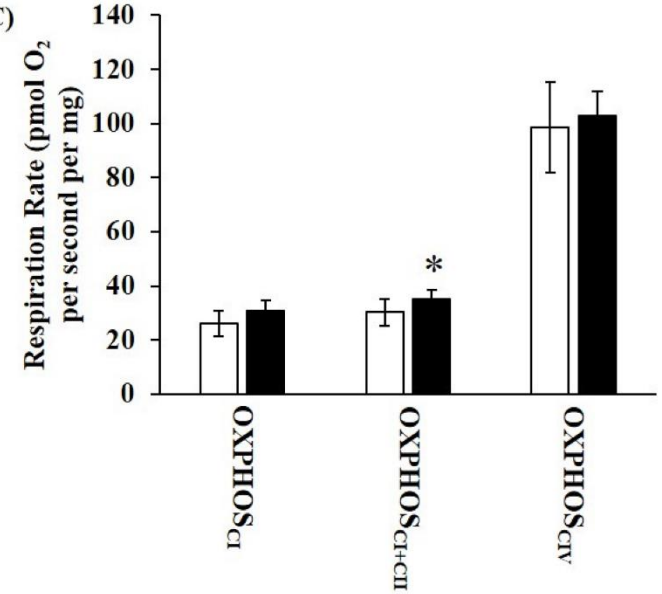
10. Iftikar, F.I., Hickey, A.J.R., 2013. Do Mitochondria Limit Hot Fish Hearts? Understanding the Role of Mitochondrial Function with Heat Stress in Notolabrus celidotus. PLOS ONE 8, e64120.
11. Johansen, J.L., Esbaugh, A.J., 2017. Sustained impairment of respiratory function and swim performance following acute oil exposure in a coastal marine fish. Aquatic Toxicology 187, 82-89.
12. Johansen, J.L., Esbaugh, A.J., 2019. Oil-induced responses of cardiac and red muscle mitochondria in red drum (Sciaenops ocellatus). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 219, 35-41.
13. Kirby, A.R., Cox, G.K., Nelson, D., Heuer, R.M., Stieglitz, J.D., Benetti, D.D., Grosell, M., Crossley, D.A., 2019. Acute crude oil exposure alters mitochondrial function and ADP affinity in cardiac muscle fibers of young adult Mahi-mahi (Coryphaena hippurus). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 218, 88-95.
14. Krumschnabel, G., Fontana-Ayoub, M., Sumbalova, Z., Heidler, J., Gauper, K., Fasching, M., & Gnaiger, E. (2015). Simultaneous high-resolution measurement of mitochondrial respiration and hydrogen peroxide production. *Methods Mol Biol, 1264*, 245-261. doi:10.1007/978-1-4939-2257-4_22
15. Mager, E.M., Esbaugh, A.J., Stieglitz, J.D., Hoenig, R., Bodinier, C., Incardona, J.P., Scholz, N.L., Benetti, D.D., Grosell, M., 2014. Acute embryonic or juvenile exposure to Deepwater Horizon crude oil impairs the swimming performance of mahi-mahi (Coryphaena hippurus). Environ Sci Technol 48, 7053-7061.
16. Mallakin, A., McConkey, B.J., Miao, G., McKibben, B., Snieckus, V., Dixon, D.G., Greenberg, B.M., 1999. Impacts of structural photomodification on the toxicity of environmental contaminants: anthracene photooxidation products. Ecotoxicology and Environmental Safety 43, 204-212.
17. Makrecka-Kuka, M., Krumschnabel, G., and Gnaiger, E., 2015. High-Resolution Respirometry for Simultaneous Measurement of Oxygen and Hydrogen Peroxide Fluxes in Permeabilized Cells, Tissue Homogenate and Isolated Mitochondria. *Biomolecules, 5*(3), 1319-1338. doi:10.3390/biom5031319
18. Pasparakis, C., Esbaugh, A. J., Burggren, W., & Grosell, M., 2019. Impacts of deepwater horizon oil on fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 224*, 108558. doi:https://doi.org/10.1016/j.cbpc.2019.06.002
19. Pesta, D. and Gnaiger, E., 2012. High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle # *T Mitochondrial Bioenergetics* (Vol. 810, pp. 25-58).
20. Ramachandran, S.D., Sweezey, M.J., Hodson, P.V., Boudreau, M., Courtenay, S.C., Lee, K., King, T., Dixon, J.A., 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Marine pollution bulletin 52, 1182-1189.
21. Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquatic Toxicology 105, 56-66.
22. Roberts, A.P., Alloy, M.M., Oris, J.T., 2017. Review of the photo-induced toxicity of environmental contaminants. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 191, 160-167.

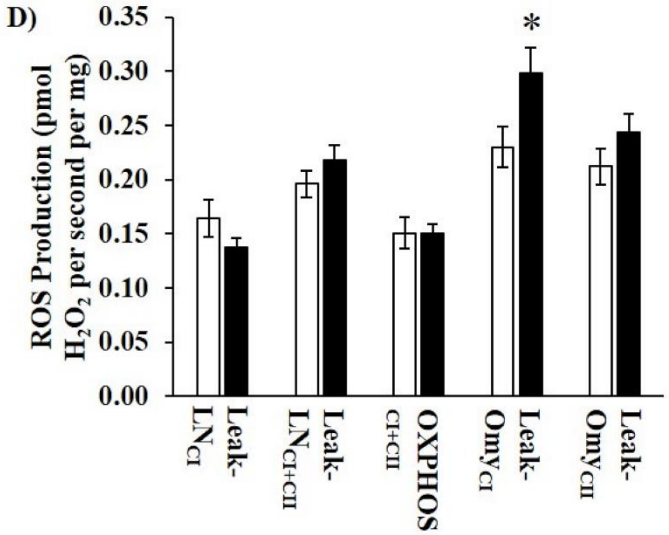
23. Sikkema, J., de Bont, J. A., & Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry, 269*(11), 8022-8028.
24. Suski, J. M., Lebiedzinska, M., Bonora, M., Pinton, P., Duszynski, J., and Wieckowski, M. R., 2012. Relation between mitochondrial membrane potential and ROS formation. *Methods Mol Biol, 810*, 183-205. doi:10.1007/978-1-61779-382-0_12
25. Wade, T.L., Sweet, S.T., Sericano, J.L., Guinasso, N.L., Diercks, A.R.R., Highsmith, R.C., Asper, V.L., Joung, D.D., Shiller, A.M., Lohrenz, S.E., 2011. Analyses of water samples from the Deepwater Horizon oil spill: Documentation of the subsurface plume. Monitoring and Modeling the deepwater horizon oil spill: a record-breaking enterprise, 77-82.
26. Weinstein, J.E., Polk, K.D., 2001. Phototoxicity of anthracene and pyrene to glochidia of the freshwater mussel Utterbackia imbecillis. Environmental Toxicology and Chemistry 20, 2021-2028.
27. Xu, E.G., Khursigara, A.J., Magnuson, J., Hazard, E.S., Hardiman, G., Esbaugh, A.J., Roberts, A.P., Schlenk, D., 2017. Larval Red Drum (Sciaenops ocellatus) Sublethal Exposure to Weathered Deepwater Horizon Crude Oil: Developmental and Transcriptomic Consequences. Environmental Science & Technology 51, 10162-10172.
28. Yin, Y., Jia, H., Sun, Y., Yu, H., Wang, X., Wu, J., Xue, Y., 2007. Bioaccumulation and ROS generation in liver of Carassius auratus, exposed to phenanthrene. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 145, 288-293.

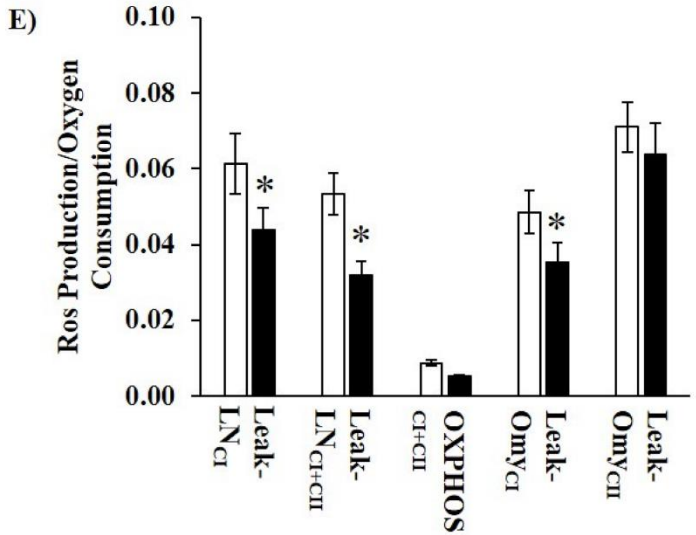
Figure

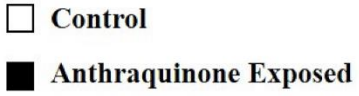

A)
1 mm


B)
Respiration Rate (pmol O2 per second per mg)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
*
LNCI
Leak-
LNCI+CII
Leak-
CI+CII
OXPHOS
OmyCI
Leak-
OmyCII
Leak-
RCR


C)
Respiration Rate (pmol O2 per second per mg)
140
120
100
80
60
40
20
0
*
OXPHOSCI
OXPHOSCI+CII
OXPHOSCIV


D)
ROS Production (pmol H2O2 per second per mg)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
LNCI
Leak-
LNCI+CII
Leak-
CI+CII
OXPHOS
OmyCI
Leak-
OmyCII
Leak-


E)
Ros Production/Oxygen Consumption
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
LNCI
Leak-
LNCI+CII
Leak-
CI+CII
OXPHOS
OmyCI
Leak-
OmyCII
Leak-


Control
Anthraquinone Exposed

Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).