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Heritable oxidative phosphorylation differences in a pollutant resistant *Fundulus heteroclitus* population

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ABSTRACT

Populations can adapt to stress including recent anthropogenic pollution. Our published data suggests heritable differences in hepatocyte oxidative phosphorylation (OxPhos) metabolism in field-caught killifish (*Fundulus heteroclitus)* from the highly polluted Elizabeth River, VA, USA, relative to fish from a nearby, relatively unpolluted reference site in King's Creek VA. Consistent with other studies showing that Elizabeth River killifish are resistant to some of the toxic effects of certain contaminants, OxPhos measurements in hepatocytes from field-caught King's Creek but not field-caught Elizabeth River killifish were altered by acute benzo [a] pyrene exposures. To more definitively test whether the enhanced OxPhos metabolism and toxicity resistance are heritable, we measured OxPhos metabolism in a laboratory-reared F3 generation from the Elizabeth River population *versus* a laboratory-reared F1 generation from the King's Creek population and compared these results to previous data from the field-caught fish. The F3 Elizabeth River fish compared to F1 King's Creek fish had significantly higher State 3 respiration (routine metabolism) and complex II activity, and significantly lower complex I activity. The consistently higher routine metabolism in the F3 and field-caught Elizabeth River fish *versus* F1 and field-caught King's Creek fish implies a heritable change in OxPhos function. The observation that LEAK, E-State, Complex I and Complex II were different in laboratory bred *versus* field-caught fish suggests that different physiological mechanisms produce the enhanced OxPhos differences. Finally, similar to field-caught Elizabeth River fish, acute benzo [a] pyrene exposure did not affect OxPhos function of the laboratory-reared F3 generation, supporting the heritability of the toxicity resistance. Overall, these results suggest that the Elizabeth River population has evolved genetic changes in physiological homeostasis that enhance routine metabolism, and we speculate that these genetic changes interact with environmental factors altering the physiological mechanisms (*e.g.,* alter LEAK, Complex I, and electron transfer system capacity) used to achieve this enhanced metabolism.

1. Introduction

Populations exposed to environmental stress (*e.g.,* pollution) have the potential to adapt when stress (i) remains constant over generations, (ii) reduces individuals' fitness, and (iii) limits survival or reproduction (Bijlsma and Loeschcke, 2005). The successful individuals that function better under stress are more likely to survive and reproduce and the traits that enhance these traits will be inherited by future generations. Therefore, after generations, populations may be predominated by the selected types, and the resulting genetic structure change constitutes the process of adaptation (Futuyma, 1986; Nacci et al., 1999). However, differentiating adaptation from acclimation (the reversible physiological changes individuals make to cope with an altered environment) can be difficult in natural populations, when the responses to an altered environment can be due to both genetic adaptation and physiological acclimation. This appears to be the case with the salt-marsh minnow, *Fundulus heteroclitus,* inhabiting highly polluted environments. *Fundulus heteroclitus* from the Elizabeth River, VA, a site highly polluted with polycyclic aromatic hydrocarbons (PAHs) (Di Giulio and Clark, 2015; Vogelbein et al., 2008; Walker et al., 2004), exhibit both heritable (Meyer and Di Giulio, 2002; Meyer

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and Di Giulio, 2003; Nacci et al., 1999; Ownby et al., 2002) and non-heritable (Meyer and Di Giulio, 2002; Meyer and Di Giulio, 2003) changes compared to *F. heteroclitus* from a nearby non-polluted reference population, King's Creek. Total PAH concentrations of 383 ug/g dry sediment were reported in the Elizabeth River, by Vogelbein et al. (2008) to the Virginia Department of Environmental Quality (Vogelbein et al., 2008). Among those PAHs, concentrations of benzo [a] pyrene (BaP), a representative PAH, were 42 ug/g dry sediment (Vogelbein et al., 2008). In contrast, King's Creek has much lower sediment PAHs, which were reported less that 1% of Elizabeth River concentrations (Clark et al., 2013; Jung et al., 2011).

We recently examined oxidative phosphorylation (OxPhos) metabolism in field-caught Elizabeth River *F. heteroclitus*. The OxPhos pathway is responsible for cellular ATP production within mitochondria and consists of five multi-subunit enzyme complexes imbedded in the inner mitochondrial membrane (Hatefi, 1985; Pagliarini and Rutter, 2013). During OxPhos, electron transfer drives complexes I, III, and IV to pump protons into the mitochondrial intermembrane space, producing a proton gradient across the inner membrane (Hatefi, 1985). Proton gradient dissipation through complex V drives ATP synthesis (Boyer, 1997; Hatefi, 1985; Schultz and Chan, 2001). However, the electron transfer and ATP synthesis processes are considered incompletely coupled since protons can also leak across the inner membrane, thus relieving the proton gradient independently of complex V (Divakaruni and Brand, 2011).

Anthropogenic pollutants have been reported to inhibit mitochondrial OxPhos functions, *e.g.* decrease ATP synthesis, inhibit electron transfer, and increase uncoupling efficiency (Chesney and Allen, 1974; Sivalingan et al., 1973; Xia et al., 2004; Zhu et al., 1995). Altered OxPhos gene expression was also detected in polluted *F. heteroclitus* populations (Fisher and Oleksiak, 2007; Oleksiak, 2008). Thus, altered OxPhos functions in pollutant-exposed fish may reflect stress responses; however, altered OxPhos responses in populations chronically-exposed to pollutants might reflect adaptive responses. Considering its crucial role in energy production and cellular functions, understanding the interactions between OxPhos and anthropogenic pollutants will help clarify the molecular basis of population-level responses to chronic pollution exposure and aid in investigating metabolic diseases.

In a prior publication, we have shown that OxPhos function is altered in field-caught *F. heteroclitus* from the polluted Elizabeth River. These fish showed higher hepatocyte OxPhos metabolism, higher coupling efficiency, and greater resistance to BaP compared to fish from a nearby, clean, reference population inhabiting King's Creek, VA (Du et al., 2015). To test the hypothesis that the altered OxPhos function in the Elizabeth River *F. heteroclitus* population is adaptive rather than a stress or acclimatory response associated with direct exposures, we compared OxPhos function in laboratory-reared, F3 *F. heteroclitus* from the polluted Elizabeth River population to OxPhos function in laboratory-reared, F1 *F. heteroclitus* from the reference King's Creek population. The retention of OxPhos traits in the 3rd generation Elizabeth River fish would eliminate epigenetic, developmental, and irreversible physiological effects, and thus would indicate that the OxPhos differences between the polluted and reference populations are heritable and most likely an evolutionary adaptation in response to anthropogenic pollution.

2. Materials and methods

2.1. Fish husbandry and treatments

Fish used were a laboratory-raised, F3 generation of *Fundulus heteroclitus* that were collected from Elizabeth River, VA (Atlantic Wood Industries site) and a laboratory-raised F1 generation of *F. heteroclitus* collected from a nearby reference site, King's Creek, VA. The Elizabeth River fish were spawned in 2011, and the King's Creek were spawned in 2012 (Table 1). All fish were provided by the US Environmental Protection Agency (EPA), Office of Research and Development, Atlantic Ecology Division, Narragansett, RI, and shipped to University of Miami in March 2014. Then fish were acclimated in re-circulating aquatic system tanks for another four months with controlled temperature (20° C) and salinity (15 ppt).

Average weights (SD) are 8.96 (2.47) and 7.98 (3.12) grams for Elizabeth River F3 and King's Creek F1 fish, respectively (Table 1). Dose effects on isolated hepatocytes were determined following intraperitoneal (i.p.) injection, because it has been widely used in various dose exposures (Ishimaru et al., 2009; Karami et al., 2011; Willett et al., 1995), and more importantly it was reported as a more effective or efficient route of exposure in ecotoxicological studies as compared to other approaches (*e.g.* intramuscular injection or oral exposure) (Gao et al., 2011; Gerasimov et al., 2000; Karami et al., 2011). Therefore, before measuring hepatocyte specific OxPhos, Elizabeth River F3 fish were dosed by i.p. injection with 50 mg/kg body weight (198.2 umol/kg) benzo [a] pyrene dissolved in corn oil with an injection volume of 5 uL/g body weight for 24 h. Control or undosed Elizabeth River F3 fish and King's Creek F1 fish were i.p. injected with corn oil only with the same injection volume of 5 uL/g body weight for 24 h. Eight fish were treated in each treatment group. Then fish hepatocytes were harvested and OxPhos function was quantified *via* high resolution respirometry. The BaP dose, 50 mg/kg body weight in the experiment, was the same dose used on field-caught Elizabeth River and King's Creek populations (Du et al., 2015). We chose this dose based on literature values (Karami et al., 2011; Willett et al., 1995) and a preliminary dose response experiment (data not shown) testing different BaP doses (0 mg/kg, 10 mg/kg, and 50 mg/kg body weight): 50 mg/kg turned out to be the most potent dose in introducing detectable OxPhos changes in hepatocytes isolated from a non-tolerant population. Experimental procedures were carried out following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

2.2. Hepatocyte isolation and permeabilization

Hepatocytes were isolated as described (Du et al., 2015). Half of the isolated hepatocyctes were saved for future gene expression analysis, and half were resuspended into Miro5 (respiration media: 0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1 adjusted with 5 N KOH) and permeabilized with digitonin for OxPhos quantification (Du et al., 2015).

2.3. High-resolution respirometry

OxPhos hepatocyte function was quantified by high-resolution respirometry with the OROBOROS Oxygraph-2k (OROBOROS instrument, Austria) as described (Du et al., 2015). Data were visualized and acquired by the software DatLab (OROBOROS instrument, Austria), and respiration was measured at 28 °C, corresponding to our previous study measuring field-caught fish. OxPhos metabolism was quantified as mean respiration rates in pmol O_2 s^{-1} ml^{-1} per million cells. Sequentially exposing hepatocytes to substrates and different inhibitors allows determinations of each complex activity in the electron transport chain (Table 2).

2.4. Statistical analyses

To investigate both acute BaP exposure effects and how laboratory raised fish compare to field caught and acclimated fish,