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Cross-resistance in Gulf killifish (*Fundulus grandis*) populations resistant to dioxin-like compounds

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ABSTRACT

The Houston Ship Channel (HSC) in Houston, Texas is an aquatic environment with a long history of contamination, including polychlorinated dibenzodioxins (PCDD), polychlorinated dibenzofurans (PCDF), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and heavy metals. Populations of Gulf killifish (*Fundulus grandis*) from the HSC have adapted to resist developmental cardiac deformities caused by dioxin-like compounds (DLCs). Contaminants in the HSC have acted as a strong selective pressure on resident Gulf killifish populations. Rapid adaptation can lead to fitness costs, some as a direct result of the mechanisms involved in the adaptive process, whereas other adaptations may be more general. To explore potential fitness costs, we evaluated two Gulf killifish populations with documented resistance to DLC-induced cardiac teratogenesis (Patrick Bayou and Vince Bayou), and one previously characterized reference population (Gangs Bayou). We also characterized a previously unstudied population from Galveston Bay as an additional reference population (Smith Point). We tested the sensitivity of F1 larvae from these four populations to two classes of pesticides (pyrethroid (permethrin) and carbamate (carbaryl)) and two model pro-oxidants (*tert*-butyl hydroquinone (tBHQ) and *tert*-butyl hydroperoxide (tBOOH)). In addition, we explored their responses to hypoxia and measured resting metabolic rates ($\dot{M}O_2$). Both adapted populations were cross-resistant to the toxicity of carbaryl and both pro-oxidants tested. There were no population differences in sensitivity to permethrin. On the other hand, one reference population (Gangs Bayou) was less sensitive to hypoxia, and maintained a lower $\dot{M}O_2$. However, there were no differences in hypoxia tolerance or resting metabolic rate between the second reference and the two adapted populations. This investigation emphasizes the importance of including multiple reference populations to clearly link fitness costs or cross-resistance to pollution adaptation, rather than to unrelated environmental or ecological differences. When compared to previous literature on adapted populations of *Fundulus heteroclitus*, we see a mixture of similarities and differences, suggesting that *F. grandis* adapted phenotypes likely involve multiple mechanisms, which may not be completely consistent among adapted populations.

1. Introduction

Much of the United States (US) coastline has been impacted by industrial activity, a major source of pollutants known to negatively impact aquatic biota. Estuarine environments often contain complex mixtures of toxicants, including high levels of polychlorinated dibenzo-dioxins and furans (PCDD/Fs) (Crawford et al., 1995), polychlorinated biphenyls (PCBs) (Connolly et al., 2000; Feng et al., 1998), polycyclic aromatic hydrocarbons (PAHs) (Walker et al., 2004) and varying combinations of other contaminants (Howell et al., 2011). Aquatic organisms can be highly sensitive to the adverse effects of PCDD/Fs, PCBs and PAHs, which have a common mode of toxicity in fish often described as dioxin-like compound (DLC) toxicity, which results in constitutive induction of the aryl hydrocarbon receptor (AHR) pathway. During sensitive stages of fish development, exposure to DLCs leads to developmental defects, including cardiac teratogenesis and associated ascites, also known as blue sac disease (Incardona et al., 2004; Safe, 1984; Timme-Laragy et al., 2007; Wassenberg et al., 2002). However, several populations of fish species, including Atlantic tomcod (*Microgadus tomcod*) (Wirgin et al., 2011), northern and southern subspecies of Atlantic killifish (*Fundulus heteroclitus*) (Arzuaga and Elskus, 2002; Wills et al., 2009, 2010b) and Gulf killifish (*Fundulus grandis*) (Oziolor and Matson, 2015; Oziolor et al., 2014), have evolved

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a resistance to otherwise toxic levels of such contaminants. Given the rapid pace of the evolutionary process in each of these cases, these findings suggest that standing genetic variation containing adaptive alleles, coupled with strong selective pressure, as the most plausible explanation for the observed rapid acquisition of resistance to PCDD/Fs, PCBs, and PAHs (Whitehead et al., 2010).

As more populations and more species are found to have adapted to anthropogenic contamination, the field of evolutionary toxicology expands (Oziolor and Matson, 2015; Wirgin and Waldman, 2004). Adaptation has been observed as increased survival of target invertebrates following multiple generations of intentional pesticide use, as well as physiological changes in populations unintentionally exposed to toxic and persistent byproducts of heavy industrial activity (Martinez and Levinton, 1996; Oziolor et al., 2014). Such "evolutionary rescue" often allows resident populations to survive an abrupt and often strong selective pressure, e.g. anthropogenic contamination, but can lead to future fitness costs for the adapted populations (Bugel et al., 2014; Harbeitner et al., 2013). To understand the biological significance of population wide adaptive responses, one must consider possible changes in the capability of resident populations to tolerate or respond to other environmental pressures (Bickham, 2011). New traits often arise alongside trade-offs in physiological function. Although a new trait may be beneficial, it can also be incompatible with other mechanisms of physiological function (Ricklefs and Wikelski, 2002). An in-depth understanding of the fitness costs associated with adaptation to contaminated environments is necessary to understand the full impacts of adaptation.

Down-regulation of the AHR pathway rescues the cardiac deformities caused by AHR-activating contaminants (Carney et al., 2004; Clark et al., 2010; Matson et al., 2008). In adapted populations of *M. tomcod*, this reduced responsiveness to DLCs results from a six base pair deletion in exon 10 of AHR2 (Wirgin et al., 2011). There is some evidence for regions of selection in *F. heteroclitus* (Reitzel et al., 2014), but the mechanism(s) or genes responsible for resistance to contaminants have not yet been identified in *Fundulus*. Mechanistically, down-regulating a complex pathway, such as the AHR, can affect related pathways and alter their efficiency (Harbeitner et al., 2013). In addition, since these contaminants have only been introduced in the past century, strong selective sweeps would be necessary for such rapid adaptation to occur (Oziolor and Matson, 2015). There is evidence that populations that have adapted by down-regulating the AHR response may have compromised population health or higher susceptibility to environmental stressors (Dey et al., 1993; Harbeitner et al., 2013). In previous investigations, populations of *M. tomcod* resistant to PCB contamination in the Hudson River were suggested to have shorter life spans than reference populations (Dey et al., 1993). Northern populations of *F. heteroclitus*, also resistant to PCBs, had higher susceptibility to oxidative stress (Harbeitner et al., 2013), as well as a down-regulated response to estrogenic agonists (Bugel et al., 2014; Greytak et al., 2010). Other investigations include possible susceptibility to fluoranthene phototoxicity and hypoxia in the same adapted population of *F. heteroclitus* from the Elizabeth River (Meyer and Di Giulio, 2003). On the other hand, that population was also less susceptible to some oxidative stress associated with PAHs (Meyer et al., 2003), and to pesticide toxicity (Clark and Di Giulio, 2012). Understanding the trade-offs that arise in these populations alongside adaptive traits may provide information about mechanisms of resistance by highlighting overlapping pathways that may compete for energy or resources (Ricklefs and Wikelski, 2002).

Recent research shows that *F. grandis* populations from the Houston Ship Channel, TX (HSC) are resistant to PCB and PAH-induced cardiac teratogenesis (Oziolor et al., 2014). The HSC, a part of the greater Galveston Bay, is a heavily industrialized environment with a variety of contaminants present, which historically have been found at toxic concentrations in this area (Lakshmanan et al., 2010; Yeager et al., 2007). Among these contaminants, PCDD/Fs, PCBs and PAHs are prevalent in biota and sediment (Aguilar et al., 2013, 2014; Subedi and Usenko, 2012), but heavy metals and pesticides are found in large concentrations as well (Anchor QEA, 2010). Besides these anthropogenic sources of environmental stress, the greater Galveston Bay area has also been impacted by an array of stressors, often causing large fish kill events (Thronson and Quigg, 2008). The heavy urbanization of the HSC and nearby areas has contributed to an increase in hypoxic events that pose a danger to resident fish populations (McInnes and Quigg, 2010). Shipping activities in the HSC, through release of ballast waters, has resulted in the introduction of a variety of algal species (Steichen et al., 2012), which in combination with the heavy eutrophication of the area, has increased the occurrence of harmful algal blooms in Galveston Bay (Steichen et al., 2012). Resident resistant Gulf killifish populations represent an environmentally relevant model for studying evolutionary responses to anthropogenic contaminants, including potential fitness costs.

The Gulf killifish (*F. grandis*) is a ubiquitous euryhaline teleost along the Gulf coast. Populations of *F. grandis* have short generation turnover times and high site fidelity, which suggests similar exposure histories over numerous generations (Nelson et al., 2014; Williams et al., 2008). As a widely distributed, and locally common teleost on the Gulf coast, with broad scientific literature coverage of the closely related *F. heteroclitus* (Burnett et al., 2007), and increasing representation in the literature itself, *F. grandis* is an ideal model for population genetic and adaptation studies in the Gulf of Mexico. In addition, it is an environmentally relevant model whose health may be indicative of the health of the larger ecosystem.

Previous research in *F. heteroclitus*, the sister species of *F. grandis*, has documented cases in which pollution adaptation to a variety of DLCs has been associated with fitness costs (Harbeitner et al., 2013; Meyer and Di Giulio, 2003). To unite the investigation of possible fitness costs in adapted populations of *F. grandis* to the very complex contamination in the HSC, it is necessary to examine the physiological response to multiple natural and anthropogenic stressors to identify potential differences in susceptibility between reference and resistant populations in Galveston Bay. Similar to Clark and Di Giulio (2012), we have selected pesticides from two classes, a pyrethroid (permethrin) and a carbamate (carbaryl), which are detoxified by cytochrome P450 1A (CYP1A), a downstream product of AHR pathway activation (Clark and Di Giulio, 2012; Tang et al., 2002). Mechanistically, a down-regulated CYP1A response associated with resistance should cause the adapted populations of *F. grandis* to be more susceptible to these pesticides, although the opposite was observed in resistant *F. heteroclitus* (Clark and Di Giulio, 2012). Despite those findings, there have been multiple differences found even between Elizabeth River adapted *F. heteroclitus* (Clark and Di Giulio, 2012) and adapted *F. heteroclitus* populations from other locations (Harbeitner et al., 2013). Thus, we chose to investigate the susceptibility of *F. grandis* populations to these two classes of pesticides to examine the potential differences or similarities between populations. In addition, we chose to use the same testing system to investigate susceptibility to oxidative stress utilizing *tert*-butyl hydroperoxide (tBOOH) and *tert*-butyl hydroquinone (tBHQ). Both of these compounds are model pro-oxidants; tBOOH is known for inducing general oxidative stress, while tBHQ is well known to induce the *Nrf2* pathway (Harbeitner et al., 2013; Meyer and Di Giulio, 2003; Meyer et al., 2003; Timme-Laragy et al., 2012). The necessity for testing oxidative stress comes both from the strong oxidative potential of some of the compounds in the HSC, such as PAHs, as well as the recalcitrant AHR2 in resistant populations, which under normal conditions is known to control the induction of the *Nrf2* pathway in fish (Harbeitner et al., 2013).

Table 1
List of locations and coordinates from which fish were collected.

Population	Acronym	Coordinates	
Smith Point	SP	29°32′37.26″N	94°47′08.12″W
Gangs Bayou	GB	29°15′30.34″N	94°54′45.00″W
Vince Bayou	VB	29°43′10.00″N	95°13′13.73″W
Patrick Bayou	PB	29°43′41.64″N	95°06′50.51″W

These mechanisms suggest a possible fitness-cost for adapted *F. grandis* populations when dealing with oxidative stress, therefore warranting investigation.

Exposure to low oxygen (hypoxia) activates the hypoxia inducible factor pathway (HIF) in vertebrates (Xiao, 2015). The HIF and AHR pathways are unified by their shared use of a molecular chaperone called the aryl hydrocarbon receptor nuclear translocator (ARNT). Briefly, both HIF and the AHR must bind to ARNT after entering the nucleus to further bind to DNA and act as transcription factors, initiating the upregulation of the genes associated with either the HIF or the AHR pathway. As such, the HIF response can reduce the activation of the AHR pathway by sequestering the available ARNT within each cell (Chan et al., 1999; Schmidt and Bradfield, 1996). On the other hand, AHR active compounds can increase HIF activity in a dose-dependent manner, which is a byproduct of the presence xenobiotic responsive elements on HIF responsive genes, to which the AHR complex can also bind (Chan et al., 1999; Fleming et al., 2009). Since adapted HSC *F. grandis* have a recalcitrant AHR response, the reduction in AHR activity could be associated with a reduced overall HIF response in adapted fish, influencing their ability to respond to hypoxia. As a robust hypoxia response is an essential trait for organisms living in an estuarine environment, and a possible lower hypoxia tolerance has been described in adapted Elizabeth River *F. heteroclitus* (Meyer and Di Giulio, 2003), we chose to test the effects of acute hypoxia on resistant HSC *F. grandis*.

With these tests, we aimed to understand whether physiological trade-offs associated with resistance exist in *F. grandis* populations living in the industrialized portion of the HSC. We were interested in trade-offs that may impede the ability of those fish to deal with environmental stressors that co-occur alongside the industrial contaminants in Galveston Bay.

2. Materials and methods

2.1. Fish acquisition and care

Fish were collected using minnow traps placed at four locations in Galveston Bay (Table 1). The location of one reference population collection site (Gangs Bayou, GB) was chosen because of previous physiological and chemical data (Oziolor et al., 2014). In addition, a second reference site (Smith Point, SP) was chosen to represent an undeveloped rural area on Galveston Bay, and because fish from this site are similarly responsive to DLCs as those from the GB reference site (Fig. S1). All fish were treated with praziquantel (PraziPro CD-22968, Aquarium Solutions, Spokane Valley, WA, USA) at 15 mL/100 gal twice, after which they were depurated and allowed to acclimate for one month prior to experimentation. Fish were maintained at 10 parts per thousand (‰) salinity in artificial salt water (ASW) prepared with Instant Ocean (Mentor, OH, USA) and maintained in a recirculating water system with mechanical, biological and UV filtration on a 14:10 light:dark cycle at approximately 25 °C. Water quality was monitored daily and weekly water exchanges were performed to ensure water quality. Feedings were performed twice daily using pellet feed (Aquamax®, Fingerling Starter 200, PMI Nutritional International, LLC, Brentwood, MO, USA) and Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA).

2.2. Larval conditioning

Fish were spawned manually by pooling oocytes with milt. Aseptic technique was used to prevent contamination between populations during spawning. After a 30 min incubation period, fertilized embryos were treated with 0.3% hydrogen peroxide (H_2O_2) for one minute to prevent fungal and bacterial infections, after which they were triple rinsed and incubated in 10‰ ASW at 28 °C at a 14:10 light:dark cycle. At 24 h post fertilization (hpf), eggs were plated on western blot paper dampened with 10‰ ASW for air incubation for 9 days. At 10 days post fertilization (dpf), embryos were immersed in water, western blot paper was removed and the plate was placed on an orbital shaker for 30 min at 55 rotations per minute (rpm) to stimulate hatching. Hatched larvae were placed in 2 L of 10‰ ASW. Larvae were fed newly hatched *Artemia franciscana* (Brine Shrimp Direct, Ogden, UT, USA) daily and 80% water changes were done every 48 h.

2.3. Chemicals and dosing

At 7 days post hatch (dph), larvae were placed in groups of 10 into 100 mL of 10‰ ASW in hexane-rinsed glass jars. After a 30 min acclimation period, the ASW was removed from each container and was replaced with 100 mL dosing solution containing DMSO (0.1%) as a carrier for permethrin (0, 25, 50, 100, 200, 400 μg/L; equivalent to a range of 0.06–1.02 μM), carbaryl (0, 5, 10, 15, 20 mg/L; equivalent to a range of 0.02–0.1 mM) or *tert*-butyl hydroquinone (0, 0.1, 0.5, 0.75, 1, 1.5, 2, 3 μM). Aqueous solutions of *tert*-butyl hydroperoxide (tBOOH) (0, 2, 3, 4, 5 mM) were also dosed similarly in 100 mL volumes, but without the addition of DMSO because of the high solubility of tBOOH in water. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 24 h of immersion in dosing solutions, larvae were screened and mortality was established by lack of movement and/or lack of heartbeat. Each jar represented a single dose and was considered as a single replicate. The following results represent a minimum of three separate experiments replicating each concentration for all populations, toxicants and doses. Each experiment is considered as $n = 1$ and comprised of 10 larvae/dose. Thus the final curves represent $n = 3$ for every dose or 30 larvae.

2.4. Loss of equilibrium

Hypoxia tolerance in *F. grandis* populations was determined by exposing fish to acute hypoxia and monitoring the amount of time until fish lost equilibrium (Ho and Burggren, 2012). Spawning, incubation and hatching was performed as above. Upon hatching, larvae from each population were housed individually at 20 °C in 5 mL of 10‰ ASW in standard 12-well cell culture plates (Falcon®, cat#353043, Corning Incorporated, NY, USA). Fish were fed twice daily to cessation, prior to a complete water change to remove food from each chamber. 24 h prior to the 15 dph time point experiments, larvae were transferred to 20 °C for acclimatization.

Loss of equilibrium (LOE) experiments were conducted in 1 mL glass vials (LOE chambers) filled with hypoxic ASW (0.28 ± 0.02 kPa) plugged with dental wax to prevent larva from accessing oxygen from the air. Hypoxic ASW was created by passing nitrogen though a glass 2000 mL Erlenmeyer flask, filled to the neck with ASW, for 24 h prior to experimentation. Oxygen partial pressure (PO_2) and temperature were continuously monitored in the flask with voltage electrodes connected to OXY-REG and TEMP-REG units, interfaced to a computer though a DAQ-M controller (Loligo Systems, Tjele, Denmark). A peristaltic pump was used to deliver hypoxic water to the LOE vial via Tygon tubing (1.14 mm i.d.) that passed through a glass chamber outfitted with an oxygen optode—PreSens Oxy-4 (Precision Sensing GmbH, Rosenberg,

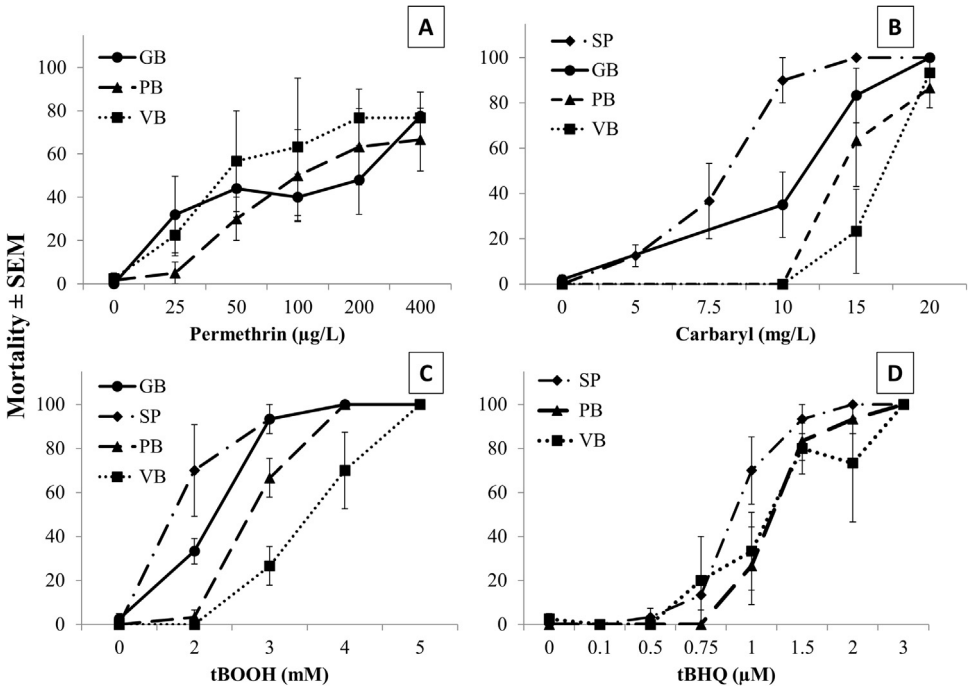

Fig. 1. Mortality dose-response to (a) permethrin (b) carbaryl (c) tBOOH (d) tBHQ of 7 days post hatch (dph) larvae of reference and adapted populations of *F. grandis*. Each point represents 30 larvae dosed in three separate experiments ($n = 3$). A: significant dose-response relationship in all populations to permethrin (1-way ANOVA, $p < 0.0001$), but no significant difference between populations (2-way ANOVA, $p = 0.3397$); B: significant dose-response relationship to carbaryl (1-way ANOVA, $p < 0.0001$) as well as a difference between populations (2-way ANOVA, $p = 0.0006$; Tukey HSD *post hoc*: SP(A); GB(AB); PB(BC); VB(C)); C: significant dose-response relationship to tBOOH (1-way ANOVA, $p < 0.0001$) and a significant difference between populations (2-way ANOVA, $p = 0.0004$; Tukey HSD *post hoc*: SP(A); GB(A); PB(AB); VB (B)); D: significant dose-response relationship to tBHQ (1-way ANOVA, $p < 0.0001$), but no significant difference between populations (2-way ANOVA, $p = 0.2906$).

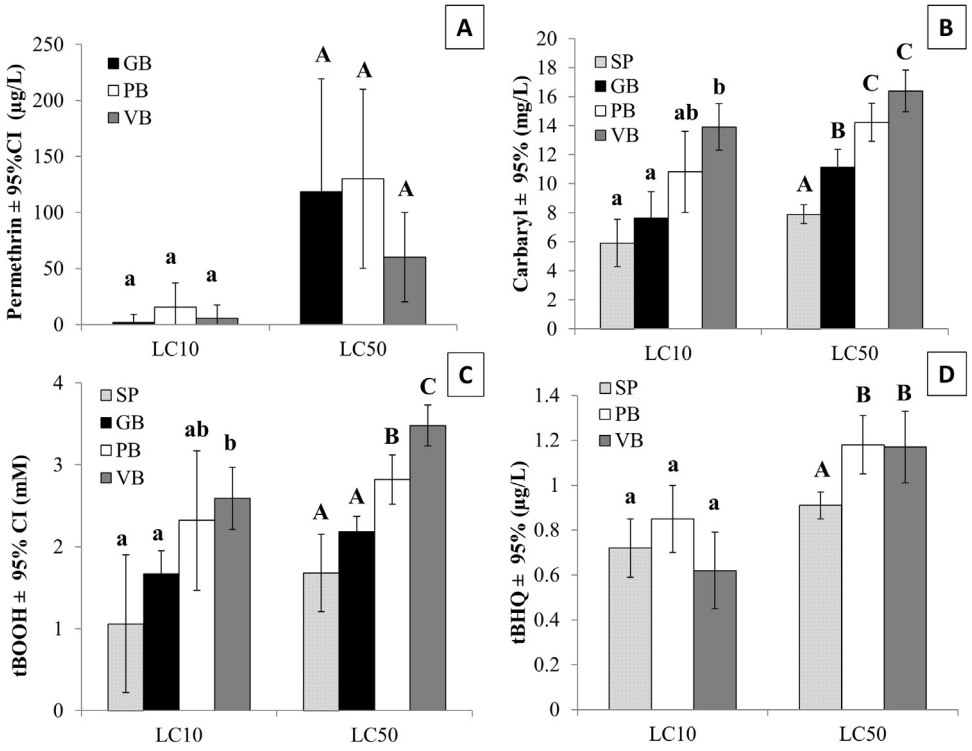

Fig. 2. Lethal concentration (LC) estimates to (a) permethrin (b) carbaryl (c) tBOOH (d) tBHQ in 7 days post hatch (dph) larvae of reference and adapted populations of *F. grandis*. Each point represents 30 larvae from three separate experiments ($n = 3$). DRC values with non-overlapping 95% confidence intervals used as an indicator of significance show that for A: permethrin there is no difference between tested populations; B: carbaryl there are significant differences between populations at both the LC_{10} and LC_{50} levels; C: tBOOH there are significant differences between populations at both the LC_{10} and LC_{50} levels; D: tBHQ there is a significant difference between populations at the LC_{50} level.

Germany), and monitored continuously to ensure oxygen tension was maintained between the flask and LOE vial.

LOE chambers were first flushed with hypoxic ASW for 30 s. Approximately 200 μL ASW was removed from the chamber prior to transferring a larva. Additional hypoxic water was then added to the chamber for 15 s (approx. 5 mL) to flush any oxygenated water, thus keeping the vial at the same oxygen tension as the source water. After which, timing began as chambers were plugged with a small ball of dental wax.

Larvae were monitored in the LOE chambers until they began to lose equilibrium, as defined by turning the LOE chamber on the anterior-posterior or left-right axis. Upon the first instance of LOE, the vial was gently inverted. Upon inversion of the vial, if the larvae responded by swimming or other movements, the vial was set down until LOE was observed again. If movement was not observed, two additional inversions were performed with five seconds between inversions. If three inversions were conducted with no response, the time was recorded as the time until loss of response (LOR). After three hours of recovery in normoxic ASW, fish were weighed, and loss of equilibrium was calculated as the LOR time per milligram fish (min/mg). A minimum of 10 fish per population were used in each experiment for a total of two independent experiments. No mortalities were observed during or within two days of experimental time points and survival to Day 15 was acceptable for all populations (GB = 95.5%; SP = 90.9%; PB = 100%; VB = 87.5%).

2.5. Metabolic rate

The rate of oxygen consumption per milligram of larvae over time was recorded to determine the resting metabolic rate ($\dot{M}O_2$) in unfed fish at rest. Larvae from each population were hatched as above and held together in 1-L polyethylene tanks at 23 °C in aerated 10‰ ASW prior to experiments. Daily 50% renewal of ASW was conducted one hour after feeding of *Artemia* nauplii, and larvae were fasted for one day prior to experiments by replacing all water and removing remaining *Artemia*.

Experiments were conducted using a 24-well plate format to continuously monitor oxygen in 3 mL chambers. Briefly, two SensorDish® Readers, each capable of simultaneously measuring oxygen in 24 chambers, were linked to allow continuous monitoring of oxygen in two 24-well OxoDish® OD24 oxygen sensor plates using SDR v38 software (Precision Sensing GmbH). Sensor Dish Readers (SDR) were installed in an incubator and temperature was maintained at the SDR at 23 °C and was continuously monitored through an integrated sensor. On the day of the experiment, fish were randomly selected from each population and randomly distributed into the chambers of the OxoDishes, with two larvae per chamber ($n \geq 6$ chambers per population) to promote mixing of oxygen and due to small size-to-chamber ratio. Chambers without larvae were filled with either ASW or anoxic water (1% $NaSO_3$ in ASW) and monitored simultaneously as standards, and experiments were conducted multiple times in the same manner.

Oxygen consumption for each chamber was calculated over time as oxygen was consumed within each chamber. $\dot{M}O_2$ was calculated as mmol of oxygen per gram per hour (Fig. 3).

2.6. Cardiac teratogenesis and EROD assay

The cardiac deformity assessment and *in ovo* EROD assays were performed as described by Oziolor et al. (2014) and Nacci et al. (1998). Briefly, embryos were fertilized as described above. They were screened at 24 hpf and placed in 100 mL hexane rinsed glass containers in pools of five with 50 mL of dosing solution. PCB126 was used as a model DLC because of its strong toxicity and AHR inducing potential. The solutions included 0.1% DMSO, PCB126 (0.01, 0.1, 0.5, 1, 5, 10, 50, 100 μg/L; equivalent to 0.03–306 nM)), and 7-ethoxyresorufin (7-ER) (21 μg/L or 0.09 μM). Embryos were incubated at 28 °C on a 14:10 light:dark cycle for 6 days.

Cardiac deformity assessment was performed as described by (Oziolor et al., 2014). Briefly, at 7 dpf embryos were screened in a blind fashion and cardiac structure was scored on a qualitative scale including normal (0), mildly deformed (1) and heavily deformed (2). A total of 10 embryos per dose were screened and each dose was repeated in three independent experiments.

In ovo EROD assay was performed as described by Nacci et al. (1998), on the same embryos assessed for cardiac deformity. Briefly, at 7 dpf embryos were screened on a Nikon AZ100 epifluorescence microscope (60× magnification; rhodamine filter; Nikon Inc.). Fluorescence intensity was measured in the urinary bladder and background fluorescence was subtracted. Fluorescence intensity quantitatively measures CYP1A enzyme activity through the metabolic conversion of 7-ER to resorufin.

2.7. Data analysis

The toxicant exposure mortality curves were compared using JMP 10 statistical software and R statistical software (R Core Team, 2015) using the *drc* bioassay analysis package (Ritz, 2005). The dose-response curves were first analyzed with a one-way ANOVA with a Tukey HSD *post hoc* test. Following this analysis, R software was used to determine which portions of the dose-response curves were driving differences between populations. Regression analysis (3 parameter, log-logistic) was performed with the *drm* function of the *drc* package. Every regression was compared to the ANOVA analysis through a lack-of-fit test and only regression models that explained more variability than the ANOVA models were used. After best fit was approved, LC_{10} and LC_{50} values were compared between populations with 95% confidence interval comparisons as indicators of significance of differences.

The loss of equilibrium dataset was analyzed by a goodness of fit test and showed a significant deviation from the normal distribution. It was further subjected to a Kruskal-Wallis non-parametric test with Dunn's *post hoc* analysis using JMP 10 statistical software.

Resting metabolic rate data were grouped by binning the data into six categories based on oxygen tension (a. 17–20 kPa, b. 14–16 kPa, c. 11–13 kPa, d. 8–10 kPa, e. 5–7 kPa, f. 0–4 kPa). Consumption data and oxygen tensions were averaged in those bins for each population and subjected to a 2-way ANOVA to determine relationship between oxygen tension and $\dot{M}O_2$, as well as differences in $\dot{M}O_2$ between populations. In addition, the top 2 bins were pooled and compared using a Kruskal-Wallis test with Wilcoxon Each Pair comparison to determine differences in $\dot{M}O_2$ between populations strictly at normoxic conditions (14–20 kPa). All $\dot{M}O_2$ data were analyzed using JMP 10 statistical software.

Cardiac deformity and EROD data were analyzed as previously described by Oziolor et al. (2014). Briefly, cardiac deformity data was rank transformed and the novel population (SP) was tested with a 1-way non-parametric (np) ANOVA with Dunnett's *post hoc* test for dose-response relationship with PCB126, a model DLC compound. Differences between populations were tested with 2-way non-parametric (np) ANOVA and Tukey HSD *post hoc* test. EROD data exhibited a Weibull distribution and was Box-Cox transformed. Dose-response to PCB126 for SP was tested with a 1-way ANOVA and Dunnett's *post hoc* test, while population differences were tested with a 2-way ANOVA and Student's *t*-test *post hoc* analysis.

3. Results

3.1. Permethrin dosing

The larval mortality experiments with permethrin showed large variability around individual concentrations for each population. The dose-response relationship between permethrin concentration and mortality was significant (1-way ANOVA; $p < 0.0001$) (Fig. 1A). However, the mortality responses of resistant populations (PB and VB) did not differ from our reference (GB) (2-way ANOVA; Tukey HSD *post hoc*: GB vs. PB, $p = 0.825$; GB vs. VB, $p = 0.597$; PB vs. VB $p = 0.311$) (Fig. 1). Similarly, the LC_{10} and LC_{50} responses did not differ significantly between populations, and had very broad confidence intervals (Fig. 2A).

3.2. Carbaryl dosing

Larval mortality dose-response curves were also significant when populations were exposed to carbaryl, and there was a significant difference in the responses between populations (2-way ANOVA; Concentration $p < 0.0001$; Population $p = 0.0006$) (Fig. 1B). In this case, one of our resistant populations (VB) had significantly lower mortality than both reference populations (SP and GB), while the other resistant population (PB) was intermediate and only significantly different from one reference (SP) (2-way ANOVA; Tukey HSD *post hoc*: SP vs. GB, $p = 0.63$; GB vs. PB, $p = 0.287$; SP vs. PB, $p = 0.0164$; SP vs. VB, $p = 0.001$; GB vs. VB, $p = 0.05$; PB vs. VB, $p = 0.850$) (Fig. 1B). The LC_{10} estimates were significantly different between GB and VB, with an intermediate and non-significant LC_{10} for PB (Fig. 2B). On the other hand, both resistant populations had significantly higher LC_{50} concentrations for carbaryl than GB, which was significantly higher than the LC_{50} value for SP.

3.3. tBOOH dosing

The dose-response relationship between tBOOH and larval mortality was significant and also significantly different among populations of *F. grandis* (2-way ANOVA; Concentration $p < 0.0001$, Population $p = 0.0004$) (Fig. 1C). Both reference populations had similar mortality curves. One of the resistant populations (VB) had significantly lower mortality than both references, while the other resistant population (PB) had intermediate, but not significantly different, mortality (2-way ANOVA; Tukey HSD *post hoc*: SP vs. VB, $p = 0.0004$; GB vs. VB, $p = 0.0065$; SP vs PB, $p = 0.073$; PB vs. VB, $p = 0.297$; GB vs. PB, $p = 0.395$; SP vs. GB, $p = 0.781$) (Fig. 1C). The LC_{10} response of one resistant population (VB) was significantly different and higher than both reference populations (SP and GB), while the other resistant population (PB) had an intermediate response and was not significantly different from either group (Fig. 2C). On the other hand, the LC_{50} responses of both resistant populations were significantly elevated relative to the reference populations. Further there were differences among the resistant populations, where the VB population had a significantly higher LC_{50} value for tBOOH than the PB population (Fig. 2C).

3.4. tBHQ dosing

There was a significant relationship between tBHQ concentration and larval mortality, but not a difference between reference and resistant populations (2-way ANOVA; Tukey HSD *post hoc*: Concentration $p < 0.0001$; Population $p = 0.29$; SP vs. PB, $p = 0.32$; SP vs. VB, $p = 0.44$; PB vs. VB, $p = 0.98$) (Fig. 1D). However, both resistant populations had significantly higher LC_{50} values than the reference (SP), whereas their LC_{10} values did not differ (Fig. 2C).

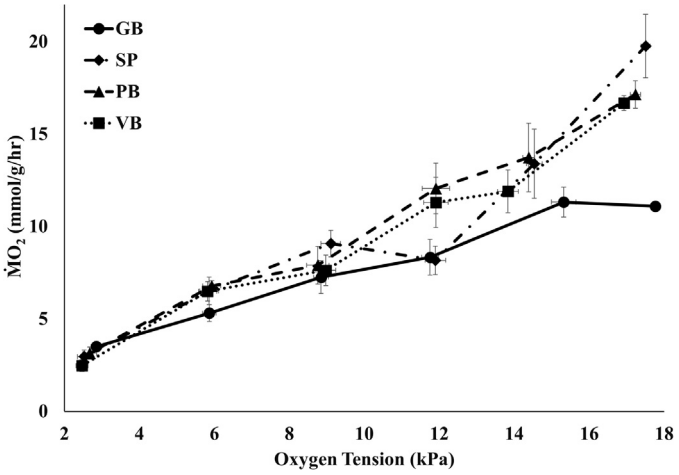

Fig. 3. Resting metabolic rate ($\dot{M}O_2$) at varying oxygen concentrations is lower in GB compared to all other sites (SP, PB, VB) (2-way ANOVA; Tukey HSD *post hoc*: GB vs PB ($p = 0.0072$), SP ($p = 0.0422$), VB ($p = 0.0441$)). A minimum of six sets of two larvae per population were tested in two separate experiments at 15 days post hatch.

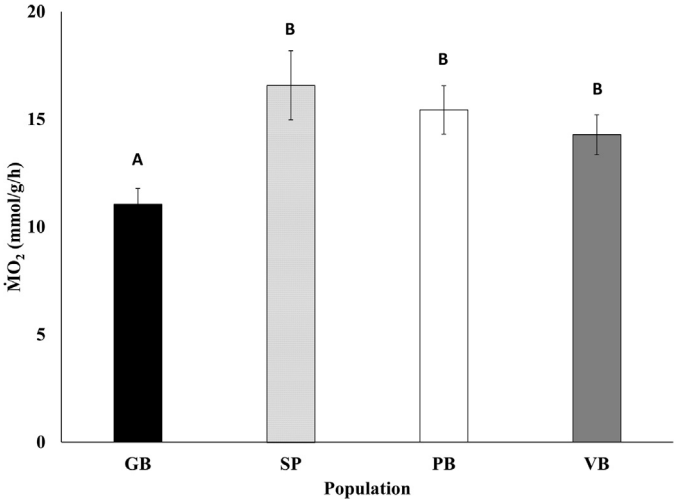

Fig. 4. Comparison of resting metabolic rate only at normoxic conditions verifies lower resting metabolic rate in GB compared to other *F. grandis* populations (Kruskal-Wallis, $p = 0.0035$; Wilcoxon each pair comparison *post hoc*: GB vs SP ($p = 0.0033$); PB ($p = 0.0095$); VB ($p = 0.0158$)). A minimum of six sets of two larvae per population were tested in two separate experiments at 15 days post hatch.

3.5. $\dot{M}O_2$ and loss of equilibrium

At two weeks post hatch, the $\dot{M}O_2$ of all four populations tested was dependent upon the amount of oxygen present in the surrounding water from normoxia through hypoxia, indicating a pattern indicative of an oxygen conformer (2-way ANOVA; $p < 0.0001$) (Fig. 3). Total oxygen consumption was significantly lower in GB larvae (reference) compared to both VB and PB resistant populations and the SP reference population (2-way ANOVA; Tukey HSD *post hoc*: GB vs. SP, $p = 0.042$; GB vs. PB, $p = 0.007$; GB vs. VB, $p = 0.044$; SP vs. PB, $p = 0.86$; SP vs. VB, $p = 1.0$; PB vs. VB, $p = 0.85$) (Fig. 3). During normoxia (14–20 kPa), the difference in $\dot{M}O_2$ had increased significance, indicating that the $\dot{M}O_2$ in GB fish is lower than that of the other populations (see below)(Fig. 4) (Kruskal-Wallis; Wilcoxon Each Pair *post hoc*: GB vs. SP, $p = 0.003$; GB vs. PB, $p = 0.01$; GB vs. VB, $p = 0.02$; SP vs. PB, $p = 0.40$; SP vs. VB, $p = 0.28$; PB vs. VB, $p = 0.37$). In addition to having a lower $\dot{M}O_2$, GB fish also had a significantly higher tolerance to hypoxic conditions, as indi-

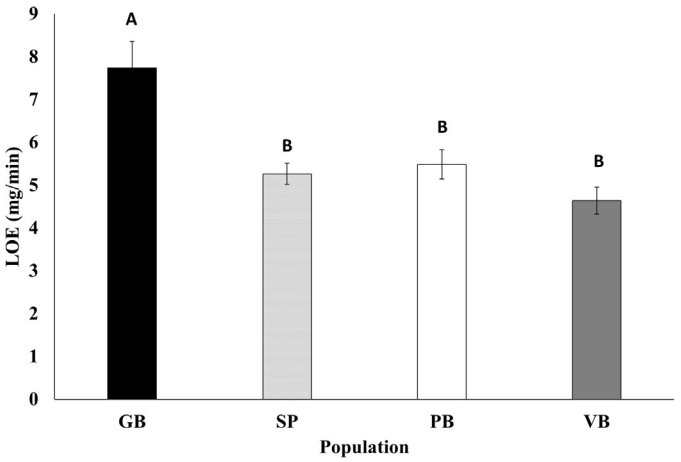

Fig. 5. GB larvae take longer to lose equilibrium when presented with hypoxic conditions (0.28 ± 0.02 kPa) (1-way ANOVA; Tukey HSD *post hoc*: GB vs SP ($p < 0.0001$); PB ($p < 0.0001$); VB ($p < 0.0001$)). A minimum 20 larvae per population were tested in two separate experiments at 15 days post hatch.

cated by the higher LOE value (Kruskal-Wallis; Dunn's *post hoc*: $p < 0.0001$; GB *p*-value < 0.01 for all comparisons) (Fig. 5). All other populations followed the same trend as for $\dot{M}O_2$ exhibiting similar times for LOE.

3.6. Cardiac deformity and EROD

The newly characterized SP population exhibited a significant dose-response relationship with PCB126, with significance at and above 1 ppb (1-way npANOVA; Dunnett's *post hoc*: $p < 0.0001$) (Fig. S1). This response was significantly different from that of both PB and VB, but not of GB (2-way npANOVA, $p < 0.0001$; Tukey HSD *post hoc*: SP vs. GB, $p = 0.92$; SP vs. PB, $p = 0.0002$; SP vs. VB, $p < 0.0001$) (Fig. S1).

The EROD activity dose-response of SP to PCB126 was also significant at every dose above control (1-way ANOVA, $p < 0.0001$; Dunnett's *post hoc*) (Fig. S1). This response was significantly different from that of PB and VB, but not GB (2-way ANOVA, $p < 0.0001$; Student's *t*-test: SP vs. GB, $p = 0.75$; SP vs. PB, $p = 0.011$; SP vs. VB, $p < 0.0001$)

4. Discussion

Anthropogenic contamination of the HSC with DLCs has led to the adaptation of *F. grandis* populations, resulting in increased resistance to the characteristic cardiovascular deformities associated with these legacy pollutants (Oziolor et al., 2014). The altered selective landscape that is necessary for rapid population adaptation can cause strong over-representation of specific genotypes associated physically with the adaptive alleles (i.e. genetic hitchhiking), loss of genetic diversity (Bickham et al., 2000) and alterations in population-wide phenotypes because of altered molecular pathways (Bickham, 2011), in this case the AHR pathway. These effects can result in populations under strong selective pressure to also present with fitness costs, defined as a lower capacity to deal with stressors of a different type (Ricklefs and Wikelski, 2002; Xie and Klerks, 2004). These fitness costs represent a physiological trade-off that is associated with the inheritance of a resistant phenotype (Ricklefs and Wikelski, 2002; Xie and Klerks, 2004). The variety of physical, chemical and biological stressors in Galveston Bay poses a threat to resident fish populations. Given the recent discovery of adapted *F. grandis* populations, it is essential to understand whether acquired resistance to DLC-induced cardiovascular teratogenesis has associated fitness costs, which may also be useful to more fully understand all of the mechanisms involved in the resistant phenotype (Thronson and Quigg, 2008).

In this investigation we found that DLC-resistant populations of *F. grandis* are also resistant to the pesticide carbaryl (Figs. 1 B and 2 B). This is similar to the findings of Clark and Di Giulio (2012), although here we showed that the resistant *F. grandis* populations from the HSC were slightly less sensitive than resistant *F. heteroclitus* larvae from the Elizabeth River in Virginia. Since carbaryl is detoxified by CYP1A, resistant populations with a recalcitrant CYP1A activity should be more sensitive to carbaryl (Oziolor et al., 2014). Carbaryl is also known to be metabolized by cytochrome p450 3A4 (CYP3A4) in human cells, leading to a different set of non-toxic metabolites (Tang et al., 2002). CYP3A4 is an enzyme regulated by both the pregnane X receptor (PXR) and the aryl hydrocarbon receptor (AHR) (Grans et al., 2014). In fish, the closest homologue to the human CYP3A4 is the CYP3A30/56 form of the enzyme that has been identified in *F. heteroclitus* (Bugel et al., 2014). So, it is not surprising that resistant *F. heteroclitus* populations utilize both PXR and CYP3A30/56, while responding to model PCBs, in contrast to reference populations (Grans et al., 2014). This finding suggests that it is possible that adapted populations of *Fundulus* may be using an alternative pathway for the biotransformation of contaminants, rather than the down-regulated AHR pathway. There have been studies that suggest resistant populations of *F. heteroclitus* display differential biotransformation of benzo[*a*]pyrene (BaP), which could lead to less toxic metabolites being produced (Wills et al., 2010a). If there is increased CYP3A30/56 activity in adapted *Fundulus*, and if it yields different metabolites for carbaryl than those produced by CYP1A, that could possibly explain the cross-resistance to carbaryl in adapted *F. grandis*. In addition, if there is an alternative detoxification mechanism, this could lead to an improved excretion efficiency for toxic metabolites and thus reduce the toxicity of the compound. Carbaryl can also be metabolized by other enzymes of the CYP family (Tang et al., 2002), leading to similar less toxic metabolites or faster clearance. Some CYPs can be activated by the PXR as well as AHR transcription factors, like CYP3s (Grans et al., 2014). Since both pathways act as defense mechanisms for detoxification and given that both PXR and AHR have a variety of ligands (Grans et al., 2014; Hahn, 2001), it is plausible that the PXR pathway could be upregulated in adapted populations to compensate for the recalcitrant AHR in adapted *F. grandis* (Oziolor et al., 2014).

Adapted populations of *F. grandis* also showed a cross-resistance to the model pro-oxidants tBOOH and tBHQ, highlighted by a lower dose-response in terms of an increased LC_{10} and LC_{50} (Figs. 1 C and D, 2 C and D, respectively). A similar cross-resistance to tBOOH was seen in adapted Elizabeth River *F. heteroclitus*, measured as differences in LT_{50} (Meyer et al., 2003). Previous studies in northern populations of *F. heteroclitus* investigating sub-lethal effects of tBHQ suggest that the higher susceptibility of these fish is a fitness-cost/trade-off that accompanies the resistance to DLCs (Harbeitner et al., 2013). A possibility for the differences in results is that the sub-lethal deformities examined as an end-point by Harbeitner et al. (2013) could be highlighting earlier developmental susceptibility of embryos, rather than an overall resistance to mortality in larvae, as examined here. On the other hand, both of the oxidative stress agents that were investigated here were, in comparison, less toxic to adapted *F. grandis* populations. This suggests that there is either a large difference in the selective pressures imposed on *F. grandis* populations from the HSC compared to northern *F. heteroclitus* populations, or a unique mode of adaptation. The latter is a hypothesis for which there is little evidence, while the former could be caused by the relatively high levels of PAH contamination in the HSC (Oziolor et al., 2014).

The increased capacity for dealing with oxidative stress poses an interesting question regarding the origin of that trait in resis-

tant populations. It has been known that PAHs, especially as studied in oil mixtures, are capable of inducing oxidative stress responses in fish, often attributed to highly redox-active metabolites (Crowe et al., 2014; Holth et al., 2014). Since PAHs are responsible for a large part of the contamination in the HSC, their presence could explain this cross-resistance to oxidative stress in adapted populations. The chronic exposure to oxidative stress could act as a selective pressure on *F. grandis* populations in the HSC. These suggestions are supported by our findings that adapted *F. grandis* populations tend to have less mortality when exposed to tBHQ (Fig. 2D), another oxidative stress agent.

Despite similarities in the cross-resistance of adapted *F. heteroclitus* and *F. grandis* populations to carbaryl, we did not observe a cross-resistance in the pyrethroid pesticide permethrin, which was seen in *F. heteroclitus* (Clark and Di Giulio, 2012). In addition to this lack of cross-resistance we have already observed a stronger resistance to carbaryl in adapted *F. grandis* populations in this manuscript, as well as a weaker resistance to PAH-induced cardiac deformities in previous studies (Oziolor et al., 2014). These data strongly suggest that there may be differences in the mechanisms through which these disparate populations of closely related species have adapted to DLC toxicity. The HSC offers a complex chemical environment comprised of PCDD/Fs, PCBs and PAHs (Anchor QEA, 2010; Howell et al., 2011, 2008; Lakshmanan et al., 2010), while the Elizabeth River is more strongly associated with PAH contamination (Landers, 2006; Walker et al., 2004). Differences in selective pressure and a majority of cross-resistance traits suggest that *Fundulus* species and populations may be adapting in mutation order manner with the common phenotype of resistance to early developmental effects of DLCs. There is evidence that supports a multi-locus adaptation in *F. heteroclitus*, which allows for the possibility of various loci contributing to the adaptive phenotype seen across *Fundulus* adapted populations (Whitehead et al., 2012).

While discussing mutation-order adaptation, it is necessary to note that there are differences between the sensitivities of these four *F. grandis* populations to carbaryl and tBOOH. In a previous study we also observed significantly lower CYP1A activity in the VB population compared to PB, while both resistant populations had significantly lower induction than the reference population (GB) (Oziolor et al., 2014). Here we also show differences between resistant populations, where VB is less susceptible to both carbaryl and tBOOH than PB. As such, the dose-response and LC_{10} of PB to carbaryl is intermediate between the references (GB, SP) and VB, while the LC_{50} is significantly higher than GB and SP (Figs. 1 B and 2 B). The same is true for the dose-response and LC_{10} for tBOOH, where PB is intermediate between the two references (GB and SP) and the cross-resistant VB (Figs. 4 and 5), while it has an LC_{50} that is significantly higher than the reference populations. This consistent intermediate response of PB compared to VB, which are ~15 km apart (shoreline distance), suggests that there may be a systematic difference between the two populations. In addition, one reference site (SP) exhibits a higher sensitivity to carbaryl compared to the other (GB) (Figs. 1 B and 2 B). One possible source of the population difference could be a lower frequency of adaptive alleles in PB, which could arise because of high immigration or maintenance of non-adaptive genotypes within the adapted population. In this case, the adaptive phenotype would be represented at a lower frequency in PB and would result in an intermediate dose-response between a population with fully fixed adaptive genotypes and reference site populations with low or no adaptive genotypes. Similarly, few adaptive genotypes in GB could explain its lower sensitivity to carbaryl compared to SP (additional difference between reference sites discussed below.) On the other hand, PB and VB may be subject to varying selective pressures because of local differences in contaminant profiles. Such differences could result in varying adaptive alleles being under selection in PB and VB, thus causing some populations to be more resistant to certain classes of contaminants (carbamates, pro-oxidants) than others. Lastly, as suggested above, it is possible that the two populations have reached a highly similar adaptive phenotype through mutation-order adaptation. If the combinations of adaptive alleles necessary for evolutionary rescue in PB and VB were different at the onset of selective pressure (~70 years ago), it is possible that the two populations fixed different combinations of adaptive alleles, leading to similar, but slightly different phenotypes. Of course, migration between these sites would likely disrupt such geographic adaptive structuring. However, it has been suggested in a recent *F. grandis* mark-recapture study that migration is quite limited for this species (Nelson et al., 2014).

Metabolic rate and responses to hypoxia were linked in these experiments, as expected. However, the pattern of effects shown was not in accordance with resistance to DLCs, or the other chemicals tested. Here, *F. grandis* from one reference population (GB) was divergent from both resistant populations (PB and VB) and also the other reference population (SP) (Figs. 3–5). GB larvae took significantly longer to reach loss of equilibrium compared to VB, PB and SP, indicating that GB larvae have an increased ability to handle hypoxia at two weeks post-hatch (Fig. 5). Not surprisingly, GB also had significantly lower overall $\dot{M}O_2$ (Fig. 3), which was most prominent under normoxic conditions (Fig. 4). This is explanatory of the increased ability to tolerate hypoxia, as the lower $\dot{M}O_2$ of GB larvae will utilize less of the available oxygen. Since the GB reference larvae were divergent from the second reference population (SP), which performed similarly to the resistant populations (VB and PB), it is most likely that the physiological phenotypes displayed in resting metabolic rate and acute response to hypoxia are independent of the mechanism of resistance to pollution identified in *F. grandis* from the HSC.

In addition to choosing two sites with little to no history of industrial pollution, we characterized both SP and GB in terms of their ability to resist cardiac teratogenesis and the inducibility of their AHR pathway, through CYP1A activity, and confirmed that both sites behave in a similar fashion, and are consistent with *F. heteroclitus* reference populations from previous studies as well (Fig. S1). Briefly, when PCB126 is present at small concentration, CYP1A activity is increased because of activation of the AHR pathway. At higher concentrations of PCB126, CYP1A activity is lower, which can be a result of onset of deformities or multiple pathways interacting because of onset of high toxicity (Fig. S1). Whereas the AHR pathway responses and sensitivity to DLCs are similar among the two reference populations, the same was not true for all of the tested endpoints. Having a reference population that behaved similarly to resistant populations, but not to another reference population, in terms of $\dot{M}O_2$ and hypoxia response, suggests that these traits are more likely to have diverged based on a common environmental variable between the three similar sites (SP, PB, VB), independent of the history of pollution shown for the resistant populations (PB, VB). In this case, we were clearly able to distinguish between what would have appeared to have been a fitness-cost (lower capacity to deal with hypoxia) if GB was the sole reference. A true reference population would be the closest possible representation of the phenotype and genotype of an adapted population before the selective pressure was introduced. There are few evolutionary toxicology studies that select multiple reference populations to confirm that observed adaptive responses and potential fitness costs or cross-resistances are functionally linked. However, our data strongly demonstrate the importance of including multiple reference sites when exploring the mechanisms and costs of anthropogenic evolutionary adaptation as done in a few previous studies (Nacci et al., 2002, 2010; Williams and Oleksiak, 2008).

Given the lack of difference found between one of the reference populations (SP) and the adapted populations (PB, VB) we have no evidence to suggest that the adaptation seen in *F. grandis* is linked to an alteration in responses to hypoxia or an increase in metabolic demand for transformation and excretion of contaminants. The comparison with the similarly responding reference and adapted populations suggests that the AHR recalcitrance seen in adapted populations does not lead to an increased susceptibility to hypoxic conditions, nor does the high contaminant load in polluted sites lead to increased metabolic demands in resident fish. The other reference population (GB) was shown to have significantly lower sensitivity to hypoxia and lower resting metabolic demand (Figs. 3–5). This suggests that the differences found between GB and all the other populations may stem from a differential environmental exposure history. It is possible that an unrelated environmental stressor has led to disparate selective pressures in GB, allowing it to diverge from other *F. grandis* populations in Galveston Bay.

The present study shows that larvae of DLC-resistant populations of *F. grandis* from the HSC also exhibit higher resistance to oxidative stress and carbamates. This result is somewhat unexpected, as based solely on AHR pathway recalcitrance, adapted populations would be predicted to exhibit increased sensitivity to both pro-oxidants and carbamate pesticides. The fact that adapted populations are actually more resistant to both suggests the involvement of multiple mechanistic pathways, thus broadening the scope of potential mechanisms involved in the adaptive resistance in these populations. Previous research has suggested a lower capacity for dealing with some stressors, such as PAH phototoxicity and hypoxia in adapted Elizabeth River *F. heteroclitus* (Meyer and Di Giulio, 2003). These data suggest that there is a large breadth of stressor responses that could be affected by the evolutionary rescue that has occurred in *Fundulus* species that are resistant to anthropogenic stressors, thus highlighting the need for an increased understanding of the genetic mechanisms responsible for the adaptation in various populations. A mechanistic approach for understanding the differential responses would add a deeper understanding of the genes affected by this adaptation and previous investigations have ventured in this area (Harbeitner et al., 2013; Meyer et al., 2003). With this manuscript we aim to provide an understanding of the responses of adapted populations of *F. grandis* to other relevant stressors, and in particular on the phenotypic differences between populations. Such information will allow for focused investigations of gene to phenotypic differences between adapted and reference populations in future experiments and elucidate the full extent of the impacts of anthropogenic contamination on both *F. grandis* and *F. heteroclitus* populations.

5. Conclusions

In this study we identified the first known instances of cross-resistance in *F. grandis* populations that are resistant to the developmental toxicity of DLCs. Here we have shown that these populations are also resistant to a carbamate pesticide (carbaryl) and two model pro-oxidants (tBOOH and tBHQ). We also identified differences between the cross-resistances found in *F. grandis* adapted populations and the ones previously described for the adapted population of *F. heteroclitus* from the Elizabeth River (Clark and Di Giulio, 2012). Thus, within *F. grandis* populations from the HSC we have identified differential sensitivity among reference populations, among adapted populations, and between reference and adapted populations. The observed complexity of sensitivity to these pesticides and pro-oxidants suggests that all of the differences in sensitivity among populations cannot be explained solely by the recalcitrant AHR response in HSC populations. In combination, the intraspecific and interspecific variation among adapted populations of *Fundulus* suggests an adaptive process that was more complex than simply the downregulation of the AHR pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.03.019.

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