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# Impact of environmental oxygen, exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17α-ethynylestradiol in the euryhaline teleost *Fundulus heteroclitus*

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ABSTRACT

17α-ethynylestradiol (EE2) is a synthetic estrogen that is an endocrine disruptive toxicant in aquatic environments. The aim of this study was to determine whether metabolic rate influenced EE2 uptake in male killifish (*Fundulus heteroclitus*), based on the hypothesis that the mechanism of EE2 uptake at the gills is similar to that of oxygen. *F. heteroclitus* were exposed to 100 ng/L radiolabeled [$^3$H]EE2 for 2 h while swimming at 0, 15, and 40 cm/s. A positive linear correlation between the rates of oxygen consumption ($MO_2$) and EE2 accumulation was seen ($r^2 = 0.99$, $p < 0.01$), with more EE2 taken up at higher swimming speeds, suggesting that oxygen uptake predicts EE2 uptake. EE2 tended to accumulate in the liver (where lipophilic toxicants are metabolized), the gall bladder (where metabolized toxicants enter bile), and the gut (where bile is received). In a subsequent experiment killifish were exposed to both hypoxic and hyperoxic conditions ($PO_2$ = 70–80 Torr, and $PO_2$ = 400–500 Torr respectively). Despite significant decreases in $MO_2$ during hypoxia, EE2 uptake rates increased only slightly with hypoxia, but in individual fish there was still a significant correlation between $MO_2$ and EE2 uptake. This correlation was lost during hyperoxia, and EE2 uptake rates did not change significantly in hyperoxia. Marked influences of salinity on EE2 uptake rate occurred regardless of the oxygen condition, with higher uptake rates in 50% seawater than in freshwater or 100% seawater. Tissue distribution of EE2 in these exposures may have been influenced by changes in tissue blood flow patterns and oxygen supply. These data will be useful in eventually constructing a predictive model to manage the optimal timing for discharge of EE2 from sewage treatment plants into receiving waters.



## 1. Introduction

Synthetic estrogens are among the most potent of toxicants in the aquatic environment, with known disruptive effects upon the endocrine systems of fish and other aquatic animals (Tyler et al., 1998). Their toxicological impact is only likely to grow, owing to an increasing prevalence in natural waters through inputs such as agricultural run-off, pulp mills and sewage treatment plants (Mills and Chichester, 2005). Of the synthetic estrogens, 17α-ethynylestradiol (EE2), used in the birth control pill and hormone replacement therapy, is considered to be a particular threat to aquatic systems. In part this is due to its hydrophobic nature and its resistance to breakdown during sewage treatment, factors that may promote enhanced environmental persistence and bioavailability (Lorphensri et al., 2007; Yamamoto et al., 2003). Furthermore, even though the human liver is capable of metabolizing EE2 into two conjugates (EE-3-O-sulfate and EE-3-O-glucuronide), the lipophilicity of these two metabolites causes them to recombine into the functional EE2 hormone upon entry into an aqueous medium (Chu et al., 2004; Tyler et al., 1998). As a result, functional EE2 is present in the aqueous environment, where it is known to affect aquatic organisms by impairing the biological actions of endogenous hormones (Hogan et al., 2010). It is well documented that in male fish EE2 exposure can induce feminization through the development of ovarian tissue and vitellogenin (Bortone and Davis, 1994; Peters et al., 2007; Purdom et al., 1994; Tyler et al., 1998), inhibition of testes growth (Jobling et al., 1995), and reduction of male hormone levels (testosterone and 11-ketotestosterone; Peters et al., 2007). Eventually effects at the whole organism level can cause population collapse (Kidd et al., 2007). In female fish, EE2 exposure causes a delay in maturation and reduction in fertility. This is evidenced by smaller eggs and gonads, as well as altered reproductive hormone levels (Werner et al., 2003).

Many sewage treatment plants are located close to the mouths of rivers, so estuaries are often receiving environments for EE2. Here not only must aquatic organisms cope with toxicants such

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as EE2, but also seasonal and daily variations in environmental factors such as temperature, salinity and dissolved oxygen. The killifish or mummichog (*Fundulus heteroclitus*) is a fish native to estuaries of eastern North America (Burnett et al., 2007). As a function of their daily tidal migrations, killifish experience fluctuations in environmental oxygen and salinity levels, as well as in their metabolic oxygen demands. These fluctuations in oxygen availability and/or uptake occur when killifish are exposed to periods of hypoxia (Nordlie, 2006), when enacting behavioral responses to tidal salinity changes (Bucking et al., 2012), and when swimming to catch prey and avoid predators (Fangue et al., 2008). As a fish's metabolic rate (MR) – the amount of energy expended in a given time – increases, its oxygen consumption ($MO_2$) also increases in order to fuel oxidative phosphorylation and ATP production (Martin and Palumbi, 1993). Thus $MO_2$ can serve as an indicator of MR (Weir, 1949). Mechanisms for increasing $MO_2$ include increases in ventilatory water flow, blood perfusion, and surface area of the gills in order to enhance oxygen diffusion from water to blood (Wood, 2001). It has been well established that the major route of uptake of organic toxicants such as EE2 is through the gills. Furthermore, we have recently shown that oxygen consumption can be used as a predictor of EE2 uptake (Blewett et al., 2013), similar to earlier findings with a variety of other lipophilic organic molecules (Brauner et al., 1994; McKim and Erickson, 1991; Murphy and Murphy, 1971; Yang et al., 2000). Ultimately, any situation that may alter the metabolic demand for oxygen will likely influence transfer of the lipophilic xenobiotics across the gills.

Our recent investigation elucidated relationships between temperature, salinity, oxygen consumption and EE2 uptake in resting killifish under normoxic conditions (Blewett et al., 2013). However, in order to better simulate conditions in nature, in the current study we altered metabolic rate via exercise and altered dissolved oxygen levels (normoxia, hypoxia, and hyperoxia) across a range of salinities to further our understanding of the relationship between oxygen consumption and EE2 uptake. We also examined the effects of these treatments on the internal distribution of EE2. We hypothesized that EE2 uptake would increase with elevated $MO_2$ during exercise. We also hypothesized that during environmental hypoxia, EE2 uptake would increase without an increase in $MO_2$ as the fish enhance the conditions for respiratory gas exchange at the gills so as to maintain $MO_2$ unchanged. Alternately, we hypothesized that during environmental hyperoxia, EE2 uptake would decrease while $MO_2$ would remain unchanged for the opposite reason. We also postulated that salinity-dependent differences in EE2 uptake established in the previous investigation (Blewett et al., 2013) would persist in the face of hypoxia or hyperoxia. Finally, we anticipated that changes in $O_2$ availability or exercise might impact the internal distribution of EE2 by altering blood flow or metabolic processing.

## 2. Methods

### 2.1. Animal husbandry

Adult male killifish (*F. heteroclitus*; mean (±S.E.M) mass of 5.86 (±0.60) g and length 9.2 (±0.2) cm), were obtained from seining at two locations: an uncontaminated site (Boudreau et al., 2005) at Horton's Creek near Miramichi, New Brunswick, Canada (47°02' N, 65°15' W) in June of 2009, and a second uncontaminated site at Shediac, New Brunswick (46°20'N, 64°40'W) in August of 2010. Fish were sexed by identification of dorsal-ventral vertical stripes along the body. Killifish were then housed at McMaster University in 400 L recirculating tanks at a salinity of 16 ppt and a temperature of 18 °C. Water was charcoal-filtered and changed every 2–3 days. Fish were placed into three groups, freshwater (FW, 0% seawater (SW)), 16 ppt (50% SW) and 32 ppt (100% SW) for three weeks prior to experimentation. To bring salinity to 16 ppt and 32 ppt, Instant Ocean salt manufactured by Aquarium Systems (Mentor, Ohio 44060, USA) was added to dechlorinated Hamilton tap water (moderately hard water: $[Na^+]$ = 0.6 mequiv/L, $[Cl^-]$ = 0.8 mequiv/L, $[Ca^{2+}]$ = 1.8 mequiv/L, $[Mg^{2+}]$ = 0.3 mequiv/L, $[K^+]$ = 0.05 mequiv/L, titratable alkalinity = 2.1 mequiv/L, pH∼8.0, hardness ∼140 mg/L as $CaCO_3$ equivalents). Fish were fed commercial nutrient flakes (Big Al's Aquarium Supercentres, Woodbridge, ON) ad libitum, and kept on an alternating light to dark cycle of 12 h per phase. All procedures were approved by the McMaster University Animal Research Ethics board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

### 2.2. Normoxia, hypoxia, hyperoxia and EE2 uptake experiments

During all exposures killifish were held in individual custom-made, shielded respirometers made of glass. These were filled with (i) FW for FW-acclimated fish, (ii) 50% SW (16 ppt) for 50% SW-acclimated fish, and iii) 100% SW (32 ppt) for 100% SW-acclimated fish. Each individual respirometer held a volume of 516 mL of water. Approximately 24 h prior to experimentation fish were placed in the respirometers and were moved to a constant-temperature water bath that maintained the experimental temperature at 18 °C via a recirculating system. The water was vigorously aerated throughout this 24 h period. This period of acclimation to the respirometers was employed to minimize the effect of stress on metabolic rate. For this 24 h and the subsequent experimental phase, fish were fasted to avoid any influence of specific dynamic action on metabolic rate.

A pilot experiment was performed with fish acclimated to 50% SW in order to select an appropriate partial pressure of oxygen ($PO_2$) range for the hypoxia experiments. After 24 h of acclimation, the water was gently replaced to avoid any disturbance. The aeration was then removed and the respirometers were sealed. A 5 mL water sample was taken to measure the $PO_2$ of the water using a Clarke-type oxygen electrode (see Section 2.2). The $PO_2$ was monitored every 30 min over 8–10 h until fish showed visible signs of distress. The rate of decrease in $PO_2$ was used to calculate $MO_2$ as a function of environmental $PO_2$ (see Section 2.5).

For normoxia experiments, the starting water $PO_2$ was approximately 150 Torr. For hypoxia and hyperoxia exposures, a water reservoir was bubbled prior to experimentation with either nitrogen or oxygen, respectively. After 24 h, the water in the respirometers was gently replaced with water at the intended experimental salinity and temperature with starting $PO_2$ of 74.8 ± 1.8 Torr for hypoxia treatments, selected on the basis of the pilot experiment. For the hyperoxia treatment, exactly the same method was used as above, except oxygen was utilized to bring water in the respirometers to a starting $PO_2$ of 428.7 ± 19.3 Torr. This level was selected as it causes a marked decline in gill ventilation and perfusion in rainbow trout (*Oncorhynchus mykiss*) (Wood and Jackson, 1980). The water was then dosed with radiolabeled [$^3$H] -17α- ethynylestradiol, obtained from American Radiolabeled Chemicals (St. Louis, MO, USA) and used at a specific radioactivity of 7,488,800 Bq/μg EE2 at a nominal exposure concentration of 100 ng EE2/L. This dose was achieved by adding an appropriate amount of non-radiolabeled EE2 in 100% ethanol (Sigma Aldrich, 98% HPLC grade, St. Louis, MO) to the radiolabeled stock. The respirometers were then closed to produce an air-tight seal. The exposure lasted for 2 h, during which 1 mL water samples were taken at 0, 60 and 120 min for radioactivity measurements, and 5 mL samples were taken at 0 and 120 min for $PO_2$ measurement. Water $PO_2$ was measured using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) connected to an AM Systems Polarographic Amplifier (Model 1900, Carlsberg, WA, USA) digital dissolved oxygen meter. The electrode was maintained and calibrated at 18 °C. At

the end of each exposure, killifish were placed in 500 mL containers with non-radiolabeled EE2 (Sigma–Aldrich, 98% HPLC grade) at a concentration of 10 μg/L for 5 min (i.e. 100× the radiolabeled exposure concentration) to displace any radiolabeled EE2 that was loosely adsorbed to the body surface. After 5 min, fish were euthanized with a lethal dose of NaOH-neutralized MS-222 (Syndel Laboratories Ltd., Vancouver, BC, Canada) and the following tissues were quickly harvested and weighed: gut, gill, liver, gall bladder, spleen and carcass (consisting of all tissues and organs not specifically excised). These tissues were then subjected to digestion and analysis of radiolabel accumulation (see below). In some experiments, the liver and gall bladder were harvested and analyzed together as a single organ.

### 2.3. Swimming experiments

Prior to experimentation, fish were fasted and housed in shielded Blazka respirometers for 24 h with constant aeration at 18 °C and 50% SW. The volume of the Blazka respirometer was approximately 3.2 L.

Sampling outlets at either end of the respirometer allowed for oxygen consumption measurements and EE2 dosing. A pump propelled water toward the rear of the chamber, creating a current for fish to swim against. A mesh screen prevented the fish from escaping the inner chamber, and plastic straws at the front of the tunnel decreased turbulence to ensure laminar flow. A black shield provided shelter and encouraged swimming further from the propeller. Fish were discouraged from resting against the mesh prior to fatigue through non-physical experimenter intervention (e.g. tapping of the swim tunnel). The 24 h fasting and settling period was implemented to eliminate error caused by stress or digestion. After this 24 h period, the respirometer was closed and dosing and sampling protocols identical to those described in Section 2.3 were followed. In preliminary trials, the maximal swim speed sustainable for 2 h by killifish was found to be approximately 40 cm/s. Thus in the EE2 uptake experiments, the fish were tested at swim speeds of 15 cm/s and 40 cm/s, as well as a resting control (0 cm/s). At the initiation of the experiment, fish were gradually brought to the desired swimming speed (15 cm/s or 40 cm/s) within 1 min after EE2 dosing.

### 2.4. Tissue analysis

All tissues were transferred to 2.0 mL graduated tubes, except for the carcass which was collected in a 50 mL Corning™ centrifuge tube. These tubes were all sealed and incubated at 65 °C for 48 h in 1 N trace metal grade $HNO_3$ (Sigma–Aldrich, St. Louis MO, USA) at a measured volume 3–5 times the mass of the organ to be digested. The gut was the exception and was digested in 2 N $HNO_3$. At 24 h, samples were removed from the incubator, vortexed and then replaced for another 24 h. Lastly, each fully digested sample was centrifuged for 5 min (carcass for 10 min) at 3500 × *g* at 18 °C. After centrifugation, the following supernatant volumes were transferred for analysis: 2 mL (carcass), 0.7 mL (gut), and 0.6 mL (other tissues). These were independently placed in 20 mL glass scintillation vials and 5 mL of the scintillation fluid Ultima Gold (PerkinElmer, Waltham, MA, USA) was added to each vial except those containing the gut and whole body, to which 10 mL was added. Radioactivity (measured in β emissions) indicative of EE2 uptake was measured using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA). Results were then quench-corrected using the external standard ratio. Water radioactivity was measured by adding 1 mL water samples to 3 mL of scintillation fluid (Opti-phase, PerkinElmer), and treating the samples similarly to the tissue samples.

### 2.5. Calculations

Calculations were performed as in Blewett et al. (2013). Briefly, $MO_2$ (μmol/5 g wet wt/h) was calculated using the following equation (Boutilier et al., 1984):

$$MO_2 = \frac{P_{O_{2i}} - P_{O_{2f}}}{\Delta t} \times V \times \alpha O_2 \times S_{Cc}$$

In the above equation, $P_{O_{2i}}$ and $P_{O_{2f}}$ are the initial and final partial pressures of oxygen (Torr), $\Delta t$ represents the total time of the experiment (h), $V$ is the volume of the respirometer (L) minus the mass of the fish (kg), $\alpha O_2$ is the $O_2$ solubility coefficient at the relevant salinity from Boutilier et al. (1984), and $S_{Cc}$ represents the mass scaling coefficient from Clarke (1999) used to scale the metabolic rate to a 5 g fish, calculated as:

$$S_{Cc} = 10^{0.79 \log(5/\text{mass}(g))}.$$

In order to quantify EE2 uptake (in ng/5 g fish/h), organ cpm (counts per minute) were corrected for individual mass, specific activity of the radioactive stock, exposure duration, and absolute water counts. More specifically, the uptake rate of each tissue was multiplied by its mass relative to that of the entire fish. These individual tissue rates were then summed to yield the total EE2 uptake rate. Since some variability in water cpm was seen between trials, EE2 uptake rates were all normalized to the nominal exposure concentration of 100 ng/L. Calculations for the total whole body uptake are expressed below:

$$\text{EE2 Uptake} = \frac{\text{CPM}_{\text{total}}}{\text{SA}} \times \frac{1}{\Delta t} \times E \times SC_c$$

In the above equation, $\text{CPM}_{\text{total}}$ is the summed EE2 counts per minute for the whole body, SA is the specific activity of the radioactive stock (cpm/ng), $\Delta t$ is the duration of exposure (h), $E$ is the ratio of 100 ng/L to the average EE2 exposure concentration over the 2 h experiment for each fish, and $Sc_c$ is the scaling coefficient used to adjust the rate to a 5 g fish (see above).

### 2.6. Statistics

All data have been expressed as the mean ± S.E.M., and where indicated, $N$ = sample size. All statistical testing was performed using the programs Sigma Plot 10.1 and SigmaStat 3.5, except for the Two-Way Analysis of Variance (ANOVA), where Statistica 10 (StatSoft) was used. Regression analyses were conducted using the linear regression function of Sigma Plot 10.1 software. Figure legends denote the specific statistical test performed in each case, but in general comparisons between experimental data points were evaluated using a One-Way or Two-way ANOVA followed by a Tukey post hoc test. If the normality test failed, a log transformation was performed which was successful in achieving normality. Data being compared were pronounced statistically significant at $p < 0.05$ and have been represented graphically such that plotted mean values that were significantly different do not share the same letter.

## 3. Results

### 3.1. EE2 uptake and oxygen consumption at different swim speeds

When swimming speed was increased from 0 cm/s to 40 cm/s, there was a significant 2.5 fold increase in oxygen consumption in killifish acclimated to 50% SW (Fig. 1A). While whole body EE2 uptake rates increased from about 0.34 ng/5 g fish/h at 0 cm/s, to 1.5 ng/5 g fish/h at 15 cm/s, and then to 8.8 ng/5 g fish/h at 40 cm/s; only the latter increase was significant (Fig. 1B). Overall, there was

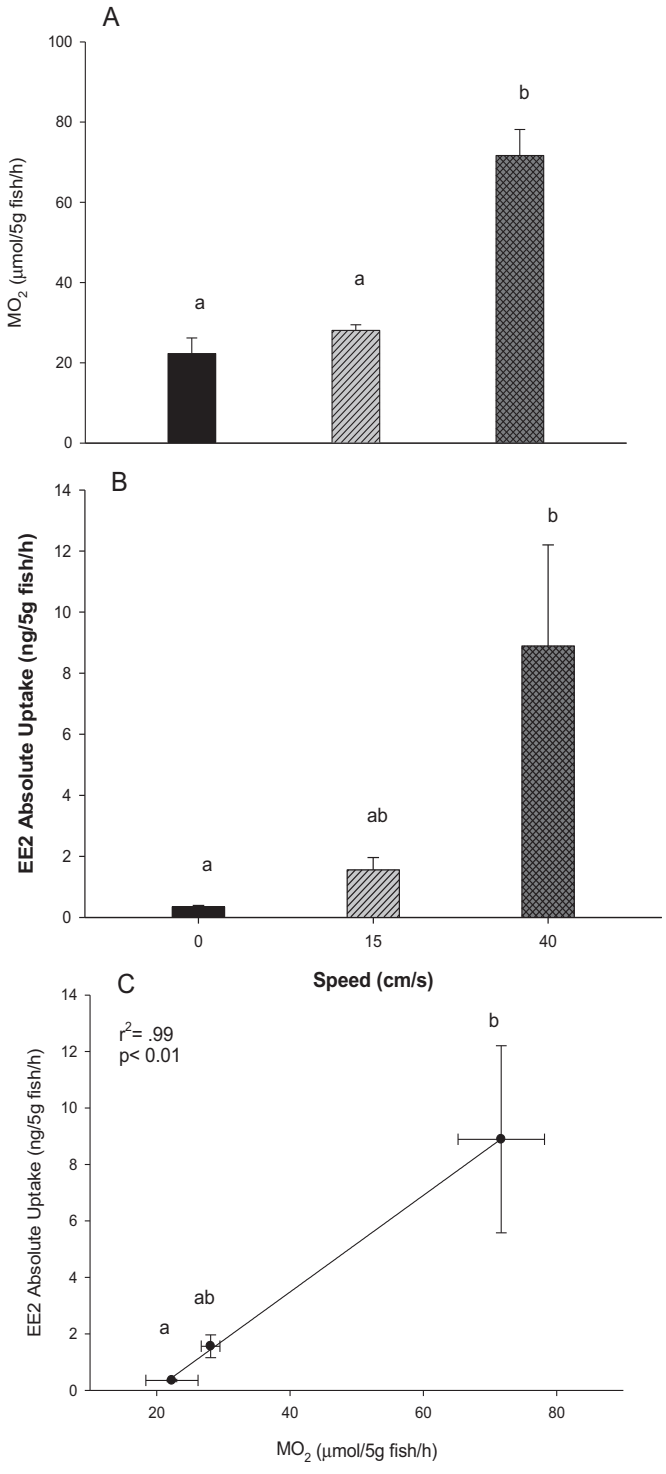


**Fig. 1.** (A). Rates of $O_2$ consumption ($MO_2$, μmol/5 g fish/h) in killifish (*Fundulus heteroclitus*) acclimated to 18 °C and 50% SW, then swum at swim speeds of 0 cm/s, 15 cm/s and 40 cm/s at the same salinity. Values are means ± S.E.M. ($N = 6$ per treatment). Means with different letters are significantly different ($p < 0.05$) as determined by a One-Way ANOVA followed by Tukey post hoc test. (B). Whole body EE2 uptake rates (ng/5 g fish/h) in killifish (*Fundulus heteroclitus*) acclimated to 18 °C and 50% SW, measured in the same treatments as panel A. Means with different letters are significantly different ($p < 0.05$) as determined by a One-Way ANOVA followed by Tukey post hoc test. (C). The overall relationship between whole body EE2 uptake rate and $MO_2$ ($r^2 = 0.99$, $p < 0.01$) in the same treatments of panels A and B.

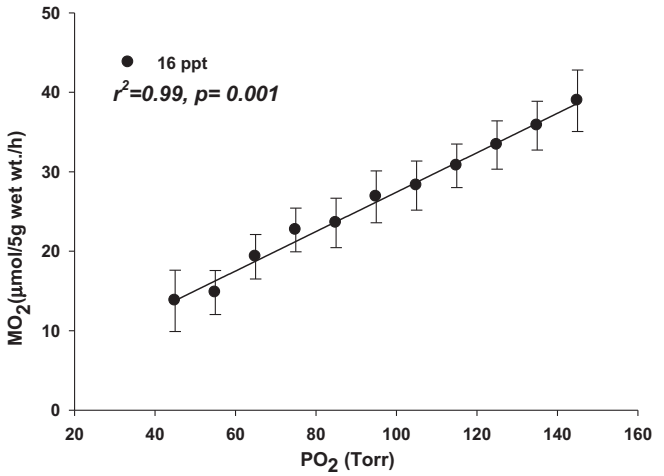


**Fig. 2.** Rates of $O_2$ consumption ($MO_2$, μmol/5 g fish/h) in killifish (*Fundulus heteroclitus*) acclimated to 18 °C and 50% SW. over decreasing environmental $PO_2$'s. Values are means ± S.E.M. ($N = 6$), $r^2 = 0.99$, $p < 0.001$.

a strong positive linear relationship between $MO_2$ and EE2 uptake ($r^2 = 0.99$, $p < 0.01$; Fig. 1C).

### 3.2. Oxygen consumption and partial pressure of oxygen

This experiment was performed with fish acclimated to 50% SW. As $PO_2$ decreased from 150 to 40 Torr, oxygen consumption steadily decreased in a close to linear fashion. The relationship between water $PO_2$ and metabolic rate displayed a strong correlation with an $r^2$ of 0.99, $p = 0.001$ (Fig. 2). Based on these data, all subsequent hypoxia experiments were started at water $PO_2$ of 70–80 Torr, a range at which $MO_2$ should be significantly reduced below normoxic levels.

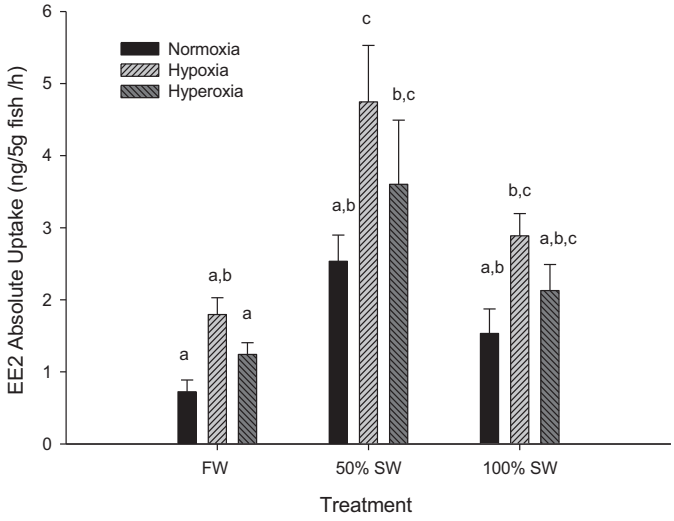


**Fig. 3.** Whole body EE2 uptake rates (ng/5 g fish/h) of killifish (*Fundulus heteroclitus*) exposed to three different salinity conditions: fresh water (FW), 50% sea water (SW, 16 ppt), and 100% SW (32 ppt) under three different dissolved oxygen levels (normoxia, hypoxia, hyperoxia; starting $PO_2$'s = 150–160 Torr, 70–80 Torr, 400–500 Torr, respectively). Values are means ± S.E.M. ($N = 6$ per treatment, except the normoxia 50% SW where $N = 4$ and the normoxia 100% SW where $N = 5$). Values not sharing the same letter are significantly different ($p < 0.05$) as determined by a Two-Way ANOVA followed by a Tukey post hoc test. Note: normoxic control data values have been previously reported in Blewett et al. (2013).

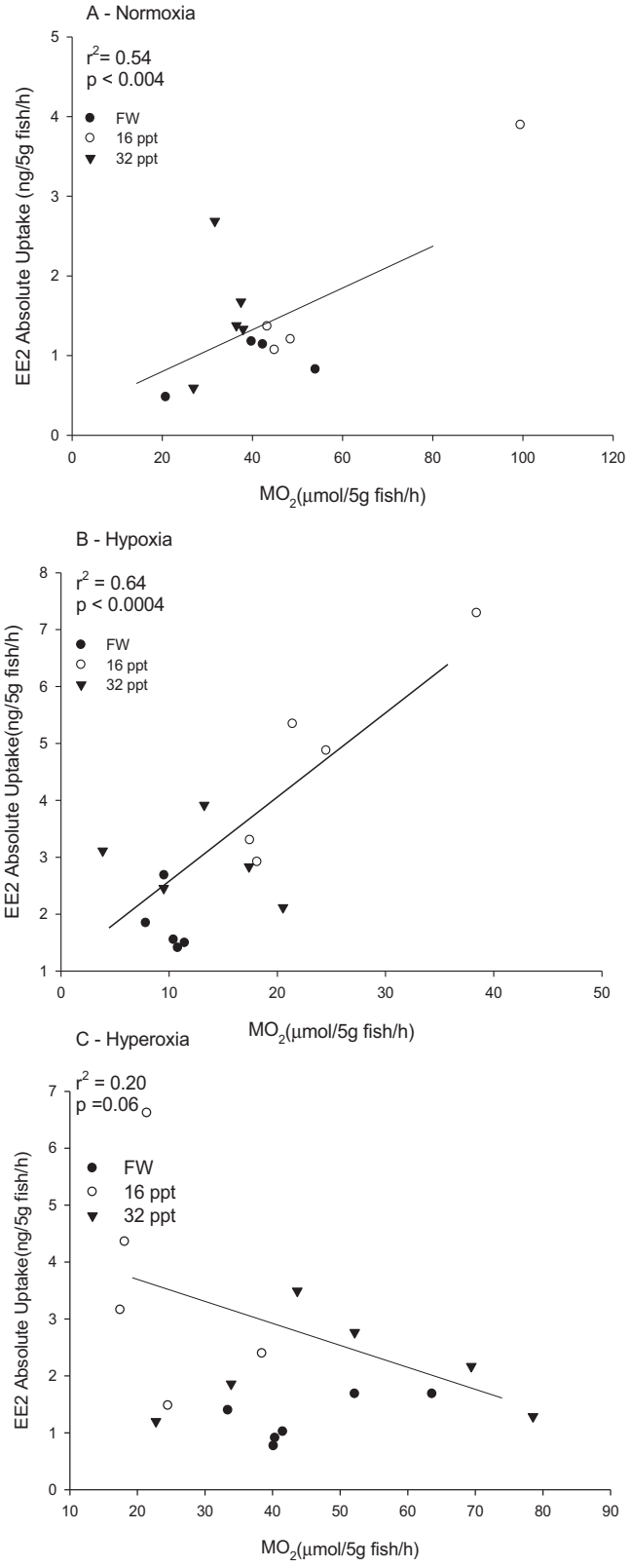


**Fig. 4.** (A) The overall relationship between whole body EE2 uptake rates and simultaneous rates of $O_2$ consumption ($MO_2$) under normoxic conditions (starting $PO_2$ = 150–160 Torr) measured in the various salinity treatments: FW, 50% SW (16 ppt), 100% SW (32 ppt) ($r^2$ = 0.54, $p$ < 0.004). $N$ = 6 per treatment, except 50% SW where $N$ = 4 and 100% SW where $N$ = 5. Values are means ± S.E.M. Note, normoxic control data values have been previously reported in Blewett et al. (2013). (B) The overall relationship between whole body EE2 uptake rates and simultaneous rates of $O_2$ consumption ($MO_2$) under hypoxic conditions (starting $PO_2$ = 70–80 Torr) measured in the various salinity treatments: FW, 50% SW (16 ppt), 100% SW (32 ppt) ($r^2$ = 0.64, $p$ < 0.0004). $N$ = 6 per treatment. Values are means ± S.E.M. (C) The overall relationship between whole body EE2 uptake rates and simultaneous rates of $O_2$ consumption ($MO_2$) under hyperoxic conditions conditions (starting $PO_2$ = 400–500 Torr) measured in the various salinity treatments: FW, 50% SW (16 ppt), 100% SW (32 ppt) ($r^2$ = 0.20, $p$ < 0.06). $N$ = 6 per treatment. Values are means ± S.E.M.

### 3.3. Absolute EE2 uptake in altered oxygen and salinity environments

Overall the Two-Way ANOVA elucidated significant effects of both salinity and environmental oxygen on EE2 uptake rate. However, the overall effect of environmental oxygen exposure level on EE2 absolute uptake rates was small. Within each tested salinity there was only a significant effect of oxygen exposure level on EE2 absolute uptake rates in the 50% SW cohort, where the hypoxia treatment exhibited a significantly elevated EE2 uptake relative to the normoxic treatment (Fig. 3). The highest uptake of EE2 occurred in the 50% SW hypoxia exposure at 4.7 ng/5 g fish/h (Fig. 3).

### 3.4. EE2 uptake and oxygen consumption as a function of dissolved oxygen levels

In individual fish, there was a strong correlation between $MO_2$ and EE2 uptake under normoxic conditions ($r^2$ = 0.54, $p$ < 0.004; Fig. 4A) for all of the salinity treatments combined. The hypoxia condition also displayed a significant positive relationship between $MO_2$ and EE2 uptake in individual fish for all salinities ($r^2$ = 0.64, $p$ < 0.0004; Fig. 4B), and in comparison to the other two treatments had significantly lower $MO_2$ values ($p$ < 0.001). In hyperoxic killifish, however, unlike the pattern seen in normoxia and hypoxia there was not a strong positive relationship between $MO_2$ and EE2 uptake. In fact the relationship was negative (a declining EE2 uptake with increasing $MO_2$ ($r^2$ = 0.20, $p$ < 0.06; Fig. 4C). Thus the clear positive relationships which were seen in normoxia and hypoxia disappeared during hyperoxia.

### 3.5. Tissue-specific accumulation of EE2 as a function of swimming speed

The tissue-specific pattern of EE2 distribution remained unchanged regardless of the swimming speed of the exposed fish (Fig. 5). For all treatments the gall bladder accumulated the largest percentage of radiolabeled EE2, with this value ranging between 30 and 40%. The carcass (~30%), the gut (~20%) and liver (~15–20%) were the next highest accumulators of EE2. At all swimming speeds both the gill and the spleen accumulated less than 5% of the total EE2 uptake (Fig. 5).

### 3.6. Tissue-specific accumulation of EE2 as a function of dissolved oxygen levels and salinity

In these experiments, the liver and gall bladder were sampled together as a single organ. In FW and 50% SW exposures, the effects of different dissolved oxygen treatments on EE2 accumulation patterns were very similar. The overall pattern was for decreased EE2 accumulation in the liver and gall bladder and increased accumulation in the carcass under hypoxia relative to normoxia or hyperoxia. In normoxia and hyperoxia the liver plus gall bladder accumulated the most EE2, followed by the carcass, gut and gill (Fig. 6A and B). Under hypoxic conditions, however, the pattern was different for both salinities (FW and 50% SW), in that the carcass displayed a significantly higher percentage EE2 accumulation relative to both normoxia and hyperoxia. This increase in accumulation was at the expense of accumulation into the liver plus gallbladder, which dropped significantly in hypoxia. There were no significant

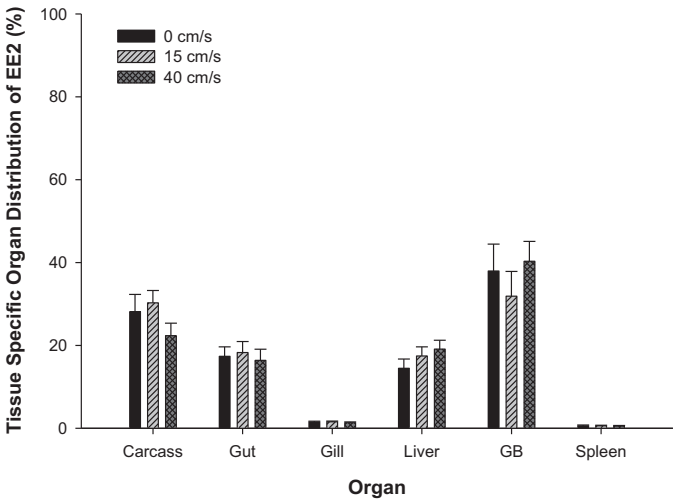


**Fig. 5.** Tissue specific organ distribution of EE2 in killifish (*Fundulus heteroclitus*) acclimated to 18 °C and 50% SW, then swum at swim speeds of 0 cm/s, 15 cm/s and 40 cm/s. Values are means ± S.E.M. ($N = 6$ per treatment). There were no significant differences for the same tissues among different swimming speeds.

changes in the gill, gut or spleen fractions as a function of dissolved oxygen (Fig. 6A and B). In killifish of the 100% SW treatment the liver plus gall bladder of the hyperoxic fish accumulated a significantly higher proportion of EE2 compared to the other two oxygen treatments. Relative to the hypoxic treatment, hyperoxic killifish accumulated a smaller proportion of EE2 in the carcass. There were no other differences in the tissue-specific pattern of EE2 uptake between dissolved oxygen treatments at this salinity. At all three oxygen levels, the liver and carcass accumulated the highest levels of EE2, followed by gut, gill and spleen (Fig. 6C).

## 4. Discussion

There were two distinct parameters examined in this study. The first was the absolute uptake of EE2, which is likely reflective of the interaction of the toxicant with the gill, the major site of uptake. The second was the tissue-specific EE2 uptake, which is more likely to reflect systemic blood flow and metabolism. The relationships of these two factors with metabolic rate appeared to vary. With regard to our original hypotheses, the expected increases in EE2 uptake were observed in killifish as metabolic rate was elevated through exercise. However, EE2 uptake rate did not increase consistently during hypoxia nor decrease during hyperoxia, in contrast to our predictions. Nevertheless, salinity-dependent differences in EE2 uptake did persist in the face of hypoxia or hyperoxia, and as predicted, changes in $O_2$ availability (but not exercise) did influence the internal distribution of EE2.

### 4.1. Increased absolute EE2 uptake as a function of swim speed

As exercise intensifies, metabolic rate increases, and therefore $MO_2$ must be elevated. Fish increase gill ventilation by increasing buccal and opercular cycling rates, and thus flush more water across the lamellae (Evans et al., 2005). This is accompanied by enhanced blood perfusion, decreased diffusion distance, and increased effective surface area for diffusion (Steen and Kruysse, 1964; Nilsson, 2007). While all of these mechanisms enhance oxygen uptake and transport, they also enhance the probability of toxicant uptake (Blewett et al., 2013; Brauner et al., 1994; McKim and Erickson, 1991; Yang et al., 2000). This is especially true for a lipophilic toxicant such as EE2, which utilizes simple diffusion to cross epithelia. Supporting this idea, both exercise (this study, Fig. 1A–C) and

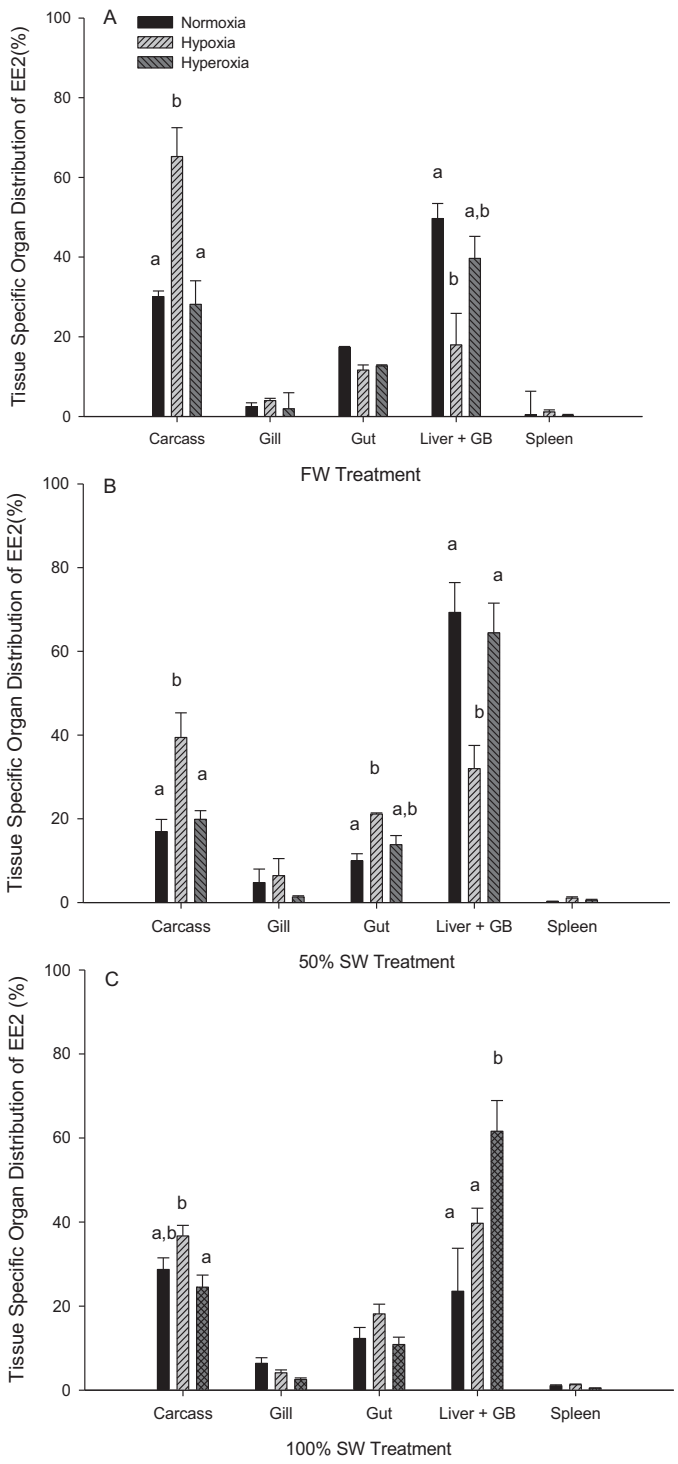


**Fig. 6.** Tissue specific organ distribution of EE2 in killifish (*Fundulus heteroclitus*) exposed to (A) FW, (B) 50% SW (16 ppt), and (C) 100% SW (32 ppt) under conditions of three different dissolved oxygen levels (normoxia, hypoxia, hyperoxia; starting $PO_2$'s = 150–160 Torr, 70–80 Torr, 400–500 Torr, respectively) at 18 °C. Values are means ± S.E.M. ($N = 6$ per treatment, except for normoxia 50% SW where $N = 4$, and normoxia 100% SW where $N = 10$). Note that the 'liver' contains both the liver and the gallbladder. Means with different letters are significantly different ($p < 0.05$) within an organ group (not across different tissues) as determined by a One-Way ANOVA followed by Tukey post hoc test.

increased temperature (Blewett et al., 2013) increase $MO_2$ and EE2 uptake via the gills.

It is notable that the absolute values of both $MO_2$ and EE2 uptake were generally lower in the swimming respirometry experiments than in other tests (c.f. Fig. 1 versus Figs. 2–4.) The reason for this is unknown. It did not appear to reflect differences in activity, but it is possible that the fish were less stressed in the swimming respirometers which had an approximately 6-fold greater volume than those used in the other trials. The fish were also collected from different sites, in two separate years, so there may have been batch differences. Nevertheless, the data were internally consistent within each series, and overall trends (i.e. $MO_2$ and EE2 uptake tending to increase in parallel) were consistent between series.

### 4.2. Oxygen consumption and partial pressure of oxygen

As the partial pressure of oxygen declined within the respirometer, the oxygen consumption of the killifish also declined. This pattern is referred to as oxyconforming (Fig. 2). While only one temperature (18 °C) was examined in the present study, in other experiments using an identical experimental regime (Blewett, MacLatchy, and Wood, unpublished data), we have seen similar patterns of oxyconforming in *F. heteroclitus* at temperatures ranging from 4 °C to 26 °C, so we consider it to be a robust result. This is a novel finding in that previous reports have placed *F. heteroclitus* as an oxyregulator (Cochran and Burnett, 1996; Richards et al., 2008; Virani and Rees, 2000), a species that maintains $MO_2$ at a constant level until a critical $PO_2$ is reached, at which point a decline in $MO_2$ occurs with declining $PO_2$. However, it is not unusual for a species to show both oxyregulation and oxyconformation. For example, this has been observed in sturgeons, where fish displayed different strategies under distinct types of stresses (Burggren and Randall, 1978; McKenzie et al., 2007). This switching of strategies is, however, unusual in that most species of aquatic vertebrates tend to oxyregulate and it is only a few species seem to have the ability to do both (Ultsch et al., 1981). Possibly oxyconforming versus oxyregulating strategies observed may be due to the experimental systems used. In our system, the animals were allowed to settle for 24 h, and then the $PO_2$ was allowed to decline slowly over 8–10 h as the fish consumed the $O_2$. This time course is generally slower than that used in most previous studies, and may have allowed the fish more time to reduce its metabolic rate in proportion to the declining $PO_2$. Another consideration is that under closed system respirometry, there is a decrease in oxygen but also an increase in carbon dioxide levels (i.e. hypercapnia), which could modify respiratory patterns, and may explain the observation of oxyconforming. However, a study by Urbina et al. (2012), found that the oxyconforming pattern in a similar euryhaline fish was not due to the effect of carbon dioxide build up within the system. Indeed, the closely related southern species of killifish, *Fundulus grandis* are reported as being oxyconformers (Subrahmanyam, 1980). Thus, further research is required to confirm if *F. heteroclitus* is a true oxyconformer. Nevertheless, having established that these killifish were oxyconformers in our experimental system, environmental $PO_2$ could be used to manipulate the pattern of $MO_2$ and thus examine the impact of a hypoxia-induced decrease in $MO_2$ on EE2 uptake rate.

### 4.3. Absolute EE2 uptake as a function of dissolved oxygen and salinity

In contrast to our original predictions, there was no consistent effect of oxygen regime on absolute EE2 uptake rates. Within salinities EE2 uptake was elevated only in our hypoxia treatment at 50% SW, and there was no significant decrease in EE2 uptake with hyperoxia, Furthermore, there was no positive relationship between EE2 and $MO_2$ in hyperoxia in contrast to the other two dissolved oxygen treatments. It has been noted that in hyperoxia, zebrafish (*Danio rerio*) display increased periods of breathing apnea (Vulesevic et al., 2005). Presumably, the larger partial pressure gradient of oxygen occurring in hyperoxia, minimizes the need to actively ventilate the gill. In the rainbow trout, hyperoxia exposure decreased gill ventilation and perfusion, and also appeared to decrease functional gill surface area (Wood and Jackson, 1980). These responses, combined with an unchanged EE2 gradient, would explain the observed effects in killifish. The level of hyperoxia used, while high (~430 mmHg; ~3 × air saturation), is environmentally-relevant. For example levels higher than 400 mmHg have been reported in estuarine rock pools (Truchot and Duhamel-Jouve, 1980).

Under the paradigm of oxygen uptake corresponding to organic toxicant uptake, it might be expected that in hypoxic conditions killifish would increase ventilation rates, re-organize gill blood perfusion and increase functional gill surface area, resulting in an increase in absolute EE2 uptake (cf. McKim and Erickson, 1991). However, this was only observed in our 50% SW treatment. This may be explained by the fact that our killifish behaved as oxyconformers and allowed their $MO_2$ to fall significantly in hypoxia (Fig. 2). This characteristic would negate the need to increase ventilation, alter gill perfusion patterns, and/or elevate gill surface area under hypoxia, and therefore would not entail an increase in EE2 uptake, nor negate the relationship of EE2 uptake with $MO_2$ in individual fish. This may also reflect the high tolerance of killifish to hypoxia. It has been shown that killifish do not avoid hypoxic waters (Wannamaker and Rice, 2000) and it has been suggested that their tolerance is aided by modification of blood oxygen binding properties that allows them to more efficiently take oxygen up from the water (Greaney and Powers, 1978).

In contrast to the limited influence of environmental oxygen levels, salinity exerted large effects on EE2 uptake rates. This marked impact of salinity on EE2 uptake has been described previously under normoxia (Blewett et al., 2013), and the same pattern may now be extended to hypoxia and hyperoxia. These authors hypothesized that this effect depended upon the gill morphology at a given salinity. For example, at 50% SW the gills of killifish do not possess any cubodial cells, characteristic of FW, and few SW chloride cells (Laurent et al., 2006). These cell types are believed to increase diffusion distances relative to pavement cells in the gill (Laurent et al., 2006; Evans et al., 2005; Perry, 1997, 1998; Wood, 2001). These changes are therefore thought to impact the diffusive uptake of EE2, such that rates will be greater in 50% SW than in FW or 100% SW. Notably, such salinity-dependent changes in gill morphology are complete within 7 days (Laurent et al., 2006), well within the three week salinity acclimation used in the current study.

### 4.4. Tissue distribution of EE2

The results of the current study support those of Blewett et al. (2013), in that the gall bladder, liver, and gut are the three tissues that showed the highest EE2 uptake, with the gall bladder containing by far the most EE2 of the three. This is consistent with other teleost research in the area of xenobiotics and hydrophobic contaminants. For example, in a study conducted on polycyclic aromatic hydrocarbons by Varanasi and Stein (1991), these toxicants tended to accumulate primarily in the liver and gall bladder. This likely relates to the mechanism of detoxification. Harmful xenobiotics such as EE2 are first transported to the liver to be metabolized by a battery of detoxification enzymes (Blom et al., 2000; Forlin et al., 1995), which would explain elevated levels of EE2 in this organ. Another reason for such a high uptake in the liver is the fact that this organ contains estrogen receptors, which would bind to a synthetic estrogen such as EE2 (Tollefsen et al., 2002; Werner et al., 2003). Furthermore, the teleost liver has a high lipid content

(Peute et al., 1978; Varanasi and Stein, 1991), rendering the liver an ideal site for accumulation of a lipophilic hormone such as EE2 (Mommsen and Walsh, 1988).

After detoxification, EE2 metabolites are transported to the gall bladder where they are incorporated into bile and are eventually excreted from the body (Lech and Bend, 1980). Researchers conducting similar work to the current study have postulated that fish swimming at faster speeds or those exposed to higher temperatures (that is, at a higher metabolic rate) would display more rapid processing of toxicants (Yang et al., 2000; Talbot and Higgins, 1982). Therefore, increased depuration resulting from increased metabolism could cause more EE2 to be transported to the liver, be detoxified, and pass through to the gall bladder. Indeed, in our previous study on *F. heteroclitus* (Blewett et al., 2013), we saw a trend for greater relative EE2 accumulation in the liver and gall bladder with increasing temperature. However, we did not see this pattern with increased swimming speed in the present study.

In contrast to the relationships observed between metabolic rate ($MO_2$) and absolute EE2 uptake, the relative uptake of EE2 into tissues did not seem to show such a strong correlation. For example, exercise resulted in no significant changes in EE2 relative distribution (Fig. 5). This is likely because once EE2 has passed the barrier of the gills, the factors that mediate movement into the tissue are not dependent upon metabolic rate. For example, metabolic rate would not be anticipated to impact the binding of EE2 to the plasma sex-hormone binding globulin (SHBG), thought to be the main transport molecule for distribution of sex steroids in blood (Miguel-Queralt and Hammond, 2008; Nagae et al., 2008). Similarly, the diffusive gradient for EE2, as well as tissue receptor distributions and affinities throughout the body, would not be expected to be sensitive to acute metabolic rate changes.

Although the tissue distribution of EE2 was not necessarily correlated with metabolic rate, during manipulation of dissolved oxygen levels there were differences in relative accumulation (Fig. 6). In FW and 50% SW these changes were restricted to the hypoxia treatment, which reduced relative EE2 accumulation in liver plus gall bladder, resulting in a reciprocal increase in the fraction in the carcass. This may reflect changes in blood perfusion patterns that are well known to occur during hypoxia, at least in some fish species (Gamperl and Driedzic, 2009). It is not known, however, how blood flow to the liver changes in hypoxia in fish, although for some species decreases to the gut are recorded (e.g. Axelsson et al., 2002). Given that these tissues are linked via the hepatic portal vein, a decrease in liver perfusion with hypoxia is possible, and this might be expected to result in a decrease in liver and gall bladder EE2 accumulation as observed in the current study. In addition, at times of reduced oxygen supply, there may be a reduction in liver metabolism, resulting in slower metabolic processing of EE2 through into the gall bladder. In 100% SW, hyperoxia caused an increase in liver plus gall bladder EE2 accumulation (Fig. 6C) so perhaps hepatic metabolism and/or blood flow was accelerated under this condition, though the mechanism remains unknown.

The carcass of the fish usually represented the second largest fraction of EE2 accumulation (as much as 25–30% of all the EE2 uptake). Miguel-Queralt and Hammond (2008) reported that animals exposed to EE2 accumulate this toxicant in the brain, reproductive tissue, and muscle tissue. These were all represented in the carcass tissue of the present study, and all are known to possess high-affinity estrogen receptors (Loomis and Thomas, 1999).

Due to the hypotonicity of fish in saline waters they must drink the surrounding water to maintain ionoregulatory balance. This could explain the relative importance of the gut in EE2 uptake (third largest fraction after liver plus gall bladder and carcass). However, previous research has found that drinking only negligibly affects EE2 gut uptake (Blewett et al., 2013). Instead, the presence of EE2 in intestinal bile is thought to account for accumulation in the gut, because bile from the gall bladder is discharged into the anterior intestine for excretion in the feces (Grosell et al., 2000).

### 4.5. Concluding remarks

Our experimental findings suggest that under normoxia there is a relationship between $MO_2$ and absolute uptake rate of EE2, likely dictated by changes in ventilatory water flow, blood perfusion, and surface area of the gills which influence the diffusive uptake from water to blood. However, EE2 uptake remains relatively unchanged during hypoxia and hyperoxia despite alterations in $MO_2$, yet salinity markedly influences EE2 uptake under all oxygen conditions, with the greatest uptake rates in 50% SW. This saline condition is thought to cause structural changes which minimize diffusion distance in the gill. Once diffusion across the gills has occurred, EE2 distribution within the body seems to be independent of metabolic rate, at least when oxygen supply is not limited. Under hypoxia, accumulation through the liver and gall bladder is reduced. This pattern of distribution within the body likely relies on blood perfusion (i.e. altered blood flow patterns), transport proteins such as SHBG and relative receptor affinities within different organs. Finally, more research must be performed to predict uptake and tissue distribution of EE2 under conditions of altered dissolved oxygen levels and exercise in combination. This will further the current model that takes into account both temperature and salinity in estuaries in order to provide knowledge for optimal timing and location of sewage outflows into these environments.

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