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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