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The effects of temperature and salinity on 17-α-ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost (*Fundulus heteroclitus*)

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ABSTRACT

The synthetic estrogen 17-α-ethynylestradiol (EE2), a component of birth control and hormone replacement therapy, is discharged into the environment via wastewater treatment plant (WWTP) effluents. The present study employed radiolabeled EE2 to examine impacts of temperature and salinity on EE2 uptake in male killifish (*Fundulus heteroclitus*). Fish were exposed to a nominal concentration of 100 ng/L EE2 for 2 h. The rate of EE2 uptake was constant over the 2 h period. Oxygen consumption rates (MO_2), whole body uptake rates, and tissue-specific EE2 distribution were determined. In killifish acclimated to 18 °C at 16 ppt (50% sea water), MO_2 and EE2 uptake were both lower after 24 h exposure to 10 °C and 4 °C, and increased after 24 h exposure to 26 °C. Transfer to fresh water (FW) for 24 h lowered EE2 uptake rate, and long-term acclimation to fresh water reduced it by 70%. Both long-term acclimation to 100% sea water (32 ppt) and a 24 h transfer to 100% sea water also reduced EE2 uptake rate by 50% relative to 16 ppt. Tissue-specific accumulation of EE2 was highest (40–60% of the total) in the liver plus gall bladder across all exposures, and the vast majority of this was in the bile at 2 h, regardless of temperature or salinity. The carcass was the next highest accumulator (30–40%), followed by the gut (10–20%) with only small amounts in gill and spleen. Killifish chronically exposed (15 days) to 100 ng/L EE2 displayed no difference in EE2 uptake rate or tissue-specific distribution. Drinking rate, measured with radiolabeled polyethylene glycol-4000, was about 25 times greater in 16 ppt-acclimated killifish relative to FW-acclimated animals. However, drinking accounted for less than 30% of gut accumulation, and therefore a negligible percentage of whole body EE2 uptake rates. In general, there were strong positive relationships between EE2 uptake rates and MO_2, suggesting similar uptake pathways of these lipophilic molecules across the gills. These data will be useful in developing a predictive model of how key environmental parameter variations (salinity, temperature, dissolved oxygen) affect EE2 uptake in estuarine fish, to determine optimal timing and location of WWTP discharges

1. Introduction

Exposure to elevated levels of natural and synthetic estrogens can cause altered endocrine function and decreased reproductive success in fish (Leblanc et al., 1997; Munkittrick et al., 1991; Munkittrick et al., 1994). Many of these natural and xenoestrogens are discharged into freshwater and estuarine systems through wastewater treatment plants (WWTP) and industrial effluents (Langston et al., 2005). Xenoestrogens are known to mimic endogenous hormones, thereby disrupting their synthesis, degradation and metabolism (Langston et al., 2005; Thorpe et al., 2003). Examples include feminization and induction of female-specific hormones in male fish exposed to synthetic estrogens (Jobling et al., 1998; Tyler et al., 1998). One such endocrine disrupting compound is the synthetic estrogen 17-α-ethynylestradiol (EE2), which mimics the effects of the natural hormone 17-β-estradiol (E2). EE2 is most commonly used in the female oral contraceptive pill and in hormone replacement therapy in menopausal women (Peters et al., 2007; Ternes et al., 1999; Thorpe et al., 2003). While EE2 surface water concentrations are markedly lower than that of E2 in the aquatic environment, EE2 has a 10–50-fold higher potency when compared to endogenous estrogens in vivo (Segner et al., 2003). EE2 also has a greater ability to be bioavailable in aquatic ecosystems due to its longer half life

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(Langston et al., 2005; Tyler et al., 1998). Indeed, in laboratory and field studies, EE2 can cause estrogenic effects when present in the range of <1–5 ng/L (Länge et al., 2001; Parrott and Blunt, 2005). However, higher levels have been documented in Canadian sewage outflows (Ternes et al., 1999) and once discharged from WWTP, they may come in contact with aquatic life (Larsson et al., 1999).

On the eastern coast of North America, many WWTP facilities discharge into estuaries and inshore regions subject to tidal and seasonal fluctuations in temperature, salinity, and dissolved oxygen. The killifish or mummichog (*Fundulus heteroclitus*) is a small, strongly euryhaline fish native to these regions where it plays an important role in the trophic dynamics of the ecosystem (Abraham, 1985; Kneib and Steven, 1978; Kneib, 1986; Valiela et al., 1977). Endocrine disrupting effects of EE2 exposure on this species are already well-documented (Hogan et al., 2010; MacLatchy et al., 2003; MacLatchy et al., 2005; Peters et al., 2007, 2009). Killifish move diurnally to and from tidal margin shallows, where salinity, temperature and dissolved oxygen are constantly varying (Burnett et al., 2007). Increased understanding of its ionoregulatory, reproductive and developmental physiology, together with rapid progress on sequencing its genome, has made *Fundulus heteroclitus* an ideal model estuarine teleost (Burnett et al., 2007) for understanding adaptations to fluctuating environmental conditions.

While there has been a vast amount of research on the mechanisms and consequences of endocrine disruption in fish by EE2 and other xenoestrogens, most of it has been performed in vitro under standardized conditions (Tyler et al., 1998). The rates and mechanisms of EE2 uptake, and the influence of environmental parameters on these processes, have received scant attention. In the present study, we focus exclusively on these latter issues, particularly the influences of temperature and salinity, because of their environmental relevance. EE2 is a very lipophilic compound, displaying an octanol-water partition coefficient ($\log K_{ow}$) of 4.12 (Yamamoto et al., 2003). This high lipophilicity suggests that the most likely mechanism and site for EE2 uptake would be diffusion through the lipid-rich gills that account for the majority of the body surface area of the fish and are directly exposed to potentially contaminated waters. Gills are only a few cells thick, making the branchial epithelium ideal for both gas exchange and the uptake of lipophilic toxicants (Brauner et al., 1994; Yang et al., 2000).

Changes in oxygen uptake require changes in gill ventilation, perfusion and functional surface area. Thus, we hypothesized that those environmental influences that affect oxygen transfer across the gills (oxygen consumption) also affect the uptake and bioaccumulation of EE2. Temperature is one such variable, so we assessed the impact of four temperatures (4, 10, 18, and 26 °C) representative of northern east coast Atlantic inshore regions on EE2 uptake, using radiolabeled EE2 for greatest sensitivity. Salinity may be another very important variable. Not only does it alter metabolic rate but it has also been documented to fundamentally change the surface structure of the gills in killifish (Copeland, 1950; Laurent et al., 2006; Scott et al., 2004), as well as to dramatically alter gill ion flux rates (Wood and Marshall, 1994; Wood and Laurent, 2003) and electrical properties (Wood and Grosell, 2008). Therefore, we assessed the influence of representative salinities for the estuarine environment (fresh water, 50% sea water, and 100% sea water) on the uptake rate of radiolabeled EE2 in *Fundulus heteroclitus*. Furthermore, drinking rate is many-fold higher in saltwater-than in freshwater-acclimated killifish (Scott et al., 2006, 2008). The possibility exists that the gut could also play a role in EE2 uptake. Indeed, there is emerging evidence that since seawater teleosts drink the medium for osmoregulatory purposes, the gut actually accounts for 40% or more of metal uptake in marine teleosts (Grosell and Wood, 2001; Wood et al., 2004; Zhang and Wang, 2007). Therefore, we also assessed the potential involvement of drinking in uptake. A benefit of the use of the radiolabeled compound was that it allowed us to assess the short-term tissue-specific disposition of EE2 after uptake.

2. Materials and methods

2.1. Fish husbandry

2.1.1. Killifish

Adult killifish, *Fundulus heteroclitus* (1–5 g), were obtained by seining from a presumed uncontaminated site (Boudreau et al., 2005), at Horton's Creek near Miramichi, New Brunswick, Canada, (47°02′N, 65°15′W), and in June of 2009 near Shediac, Bay of Fundy, NB (46°20′N, 64°40′W) in August of 2010. Fish were then transferred to McMaster University (Hamilton, Ontario (ON), Canada) and held in 400 L aquaria. Only male killifish were used in the exposures; they were sexed by observing the presence of large blue stripes down the ventral axis while females have large white "bellies" and brown colouration. Killifish were acclimated to either 50% sea water (16 ppt, the reference condition), fresh water (FW; 0% seawater) or 100% sea water (32 ppt) at 18 °C for at least 3 weeks prior to experimentation. Fresh water was dechlorinated city of Hamilton tap water (moderately hard: $[Na^+]$ = 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; titration alkalinity 2.1 mequiv/L; pH ~ 8.0; hardness ~ 140 mg/L as $CaCO_3$). Saline waters were made by the addition of Instant Ocean salt (Big Al's Aquarium Supercenter, Woodbridge, ON) to fresh water. All aquaria were set up with re-circulating pumps that flowed water through charcoal filters. Aquarium water was changed every 2 to 3 days. Fish were fed to satiation once daily with Big Al's commercial nutrient flakes (Big Al's Aquarium Supercenter) and frozen brine shrimp (San Francisco Brand, Newark, CA, USA) and were subjected to a 12 h light:12 h dark daily photoperiod. All procedures were approved by the McMaster University Animal Research Ethics board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. Oxygen consumption and EE2 uptake experiments

During exposures killifish were held in individual custom-made, shielded respirometers, filled initially with (i) reference condition water (16 ppt at 18 °C) for most experiments or (ii) fresh water (FW) at 18 °C for FW-acclimated fish, or (iii) for salinity transfer experiments only, killifish were taken from the reference condition acclimation tank and placed in FW or in 32 ppt for 24 h prior to experimentation. An additional exposure was also performed where fish acclimated to 16 ppt at 18 °C were terminally euthanized with a lethal dose of NaOH-neutralized MS-222 30 min prior to experimentation. Euthanized fish were then placed in individual respirometers (16 ppt, 18 °C), and used as zero respiration controls to account for any non-specific binding or passive diffusion. In this experiment only, continuous aeration was provided throughout the EE2 exposure period to ensure thorough mixing. Each individual respirometer held a volume of 516 mL of water. Once fish were placed in the respirometers, the units were moved to a constant-temperature water bath at the intended experimental temperature. The temperature was controlled by a recirculating system, such that the bath holding the respirometers was connected to a constant temperature reservoir. Therefore, the water was gradually equilibrated over a 24 h period from 18 °C to the intended experimental temperature that ranged from 4 °C to 26 °C. The water was vigorously aerated throughout this adjustment period. Fish were also fasted during this 24 h period to avoid any influence of specific dynamic action (i.e. the stimulatory effect of feeding) on metabolic rate.

After 24 h, the water was gently replaced (i.e. with minimal disturbance to the fish) with water at the experimental temperature, and the aeration stone was removed. The water was then dosed with radiolabeled [^{3}H]-17-α- ethynylestradiol, obtained from American Radiolabeled Chemicals (St. Louis, MO, USA) and used at a specific radioactivity of 7488800 Bq/μg EE2 and a nominal exposure concentration of 100 ng EE2/L for each individual respirometer. This 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. In the traditional manner for radiotracer flux experiments, the uptake of total EE2 (radiolabeled plus non-radiolabeled) was calculated from the known specific activity. 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 the measurement of the partial pressure of oxygen (PO_2). In preliminary experiments with more frequent sampling, it was found that the decline in PO_2 was linear over this time period. 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, prior to radioactivity analyses: carcass, gut, gill, liver, gall bladder and spleen. In some experiments, liver and gall bladder were harvested and analyzed together as a single organ.

2.3. Chronic killifish exposure experiment

Killifish were taken from reference condition water (i) and placed into two separate 38 L tanks. Fish were allowed to acclimate in the tanks over a 24 h period prior to experimentation to allow adjustment to new surroundings. The first tank was dosed with an appropriate volume of 100% ethanol vehicle (Sigma Aldrich, 98% HPLC grade) and the second tank was dosed with non-radiolabeled EE2 in ethanol (Sigma Aldrich, 98% HPLC grade). Each tank housed 9 fish and exposures ran for 15 days; both tanks were aerated throughout the exposure, and 80% of the water was replaced daily. At the end of the 15 day exposure, fish were placed in the individual respirometers and a 2 h acute exposure with radiolabeled EE2 and oxygen consumption experimentation was performed as described above.

2.4. Time trial exposure experiments

To verify that EE2 uptake over the 2 h exposure period was linear with time, a serial sampling experiment was performed using a 38 L aquarium filled with reference condition water (i). The water had been dosed with 100 ng/L radiolabeled EE2 and continually aerated for 24 h. At the end of this time period, 24 killifish were placed in the aquarium, and 6 fish were removed, sacrificed, and sampled (as described in Section 2.2) at each of 30, 60, 90, and 120 min of exposure.

2.5. Drinking rate experiments

In separate experiments, killifish from two of the acclimation conditions (16 ppt and FW) were placed in static aerated 200 mL plastic containers (shielded) at 18°C for 8 h in the previously-mentioned water bath system. After a 2 h settling period, a dose of 8 μCi radiolabeled [^{3}H]-polyethylene glycol, M.W. 4000 (PEG-4000; Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) with a specific activity of 47360000 Bq/g was added, and the exposure continued for 6 h. Water samples (5 mL) were taken at 0, 3 and 6 h, following which fish were euthanized with a lethal dose of NaOH-neutralized MS-222. The gastrointestinal tract was then exposed via a mid-ventral incision, and the gut was tied at both the anterior and posterior ends with Ethicon™ braided silk 2.0 (3.0 metric; North Ryde, NSW, Australia) to prevent any loss of contents. The entire gut was then removed, weighed and processed for radioactivity analysis, as was the carcass.

2.6. Tissue analyses

The same methods were used for both radiolabeled [^{3}H]-EE2 and radiolabeled [^{3}H]-PEG-4000. Each organ was weighed; the carcass was placed in a 50 mL Corning™ centrifuge tube while all other organs were placed in 2 mL bullet tubes. Carcass, gill, liver, gall bladder and spleen tissues were then digested with 1 N trace metal grade nitric acid (Sigma-Aldrich) at volumes of 3–5 times (exact volume recorded) the weight of the organ, except for the gut which was digested in 2 N trace metal grade nitric acid. The sealed vials were placed in an incubator at 65 °C for 48 h, with vigorous vortexing at 24 h. The digested samples were then centrifuged for 5 min at 3500 rpm at 18 °C. The following supernatant volumes were taken for analysis: carcass 2 mL, gut 0.7 mL and the remaining tissues 0.6 mL, and were added to either 10 mL (for gut and carcass) or 5 mL (for other tissues) of scintillation fluid (Ultima Gold, Perkin Elmer, Waltham, MA). The tissue samples were counted on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer), using the external standard ratio method for quench correction. Samples were standardized to a common counting efficiency (the same as that of water samples) using a quench curve constructed from various amounts of tissue digest.

2.7. Water analyses

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 the chosen experimental temperature. Water radioactivities of either [^{3}H]-EE2 or [^{3}H]-PEG-4000 were measured by adding 1 mL water samples to 3 mL of scintillation fluid (Optiphase, Perkin Elmer), and counting on the Tri-Carb 2900TR Liquid Scintillation Analyzer, with all values quench-corrected to a common counting efficiency.

2.8. Calculations

Oxygen consumption (MO_2) was calculated using the equation below where change in water PO_2 per unit time was multiplied by the O_2 solubility coefficient (Boutilier et al., 1984), then factored by respirometer volume and normalized to a 5 g fish.

$$MO_2 = \frac{[PO_2]_I - [PO_2]_F}{\Delta t} \times V \times S_c \times Sc \quad (1)$$

where $[PO_2]_I$ and $[PO_2]_F$ are the initial and final partial pressures of oxygen (mmHg), Δt represents the time period (h), V is the volume of the respirometer (L), S_c is the solubility coefficient (μmol/L/mmHg), and Sc represents the mass scaling coefficient taken from Clarke (1999) which was calculated as $10^{0.79 \log (5/\text{weight(g)})}$. Thus MO_2 was normalized in each trial to represent a 5 g fish, and expressed as μmol/5g-fish/h, so as to remove the allometric effect of body mass on metabolic rate.

EE2 uptake rates were calculated from the counts per minute of the individual organs (CPM), mean specific activity (SA), fish weight and experimental time, and expressed in ng/g wet wt/h. In

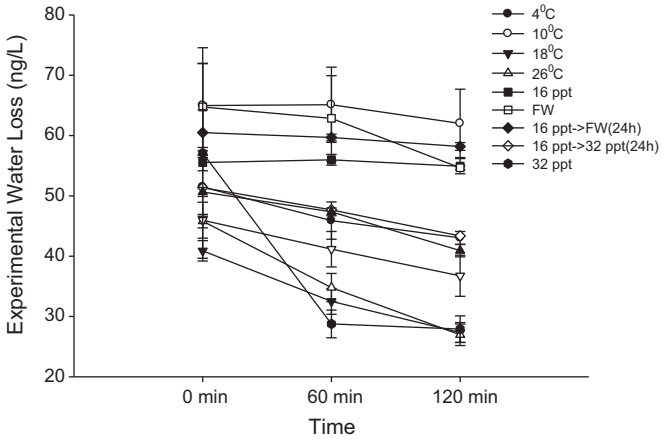

Fig. 1. Mean EE2 concentrations in ng/L, calculated from water radioactivity in the various treatments, over the course of the 2 h exposures. The original addition of radiolabeled EE2 was sufficient to create a nominal concentration of 100 ng/L, so there was a large initial loss prior to the 0 min sample. Means ± S.E.M (N = 4–6 per treatment, except for 32 ppt, where N = 10).

practice, because EE2 is notoriously "sticky" to the walls of containers (Walker and Watson, 2010), the absolute measured exposure levels were less than the nominal value of 100 ng/L and declined with time, with some variability between trials (see Fig. 1). To correct for this, all rates were adjusted to an EE2 exposure concentration of 100 ng/L, the original concentration added to each exposure tank, by the following equation:

$$\text{EE2 Uptake} = \frac{\text{CPM}_{\text{total}}}{\text{SA}} \times \frac{1}{W}\frac{1}{\Delta t} \times Z \quad (2)$$

Where $\text{CPM}_{\text{total}}$ is total counts per minute of EE2 in the whole body of the fish, W is the weight of the fish (g), SA is the specific activity of the radioactive stock (in cpm/ng), Δt is the total time of the exposure, and Z is the ratio of 100 ng/L to the mean measured EE2 exposure concentration averaged over the 2 h period for each individual fish.

Similar to MO_2, these EE2 uptake rates were also normalized to a 5 g fish using the same scaling coefficient for consistency, resulting in units of ng/5g-fish/h as follows:

$$\text{EE2 Absolute Uptake} = \text{EE2 Uptake} \times \text{Sc} \quad (3)$$

Q_{10} values for MO_2 or EE2 uptake were calculated as:

$$Q_{10} = \frac{(R_1)^{10/(t_2 - t_1)}}{(R_2)} \quad (4)$$

where R_1, and R_2 are rates of oxygen consumption or EE2 uptake at temperatures of t_1 and t_2, respectively (Schmidt-Nielsen, 1997).

Drinking rate (DR, in mL/kg/h) was calculated by taking into account the average PEG-4000 radioactivity measured in the exposure water and the total radioactivity measured in the digestive tract of individual killifish at the end of the 6 h period:

$$\text{DR} = \frac{\text{CPM}_{(\text{total gut})}}{\text{CPM}_{(\text{water})}} \times \frac{1}{\Delta t} \times \frac{1}{W} \quad (5)$$

where $\text{CPM}_{(\text{total gut})}$ is the total amount of counts per min present in the gut, Δt is the flux time, $\text{CPM}_{(\text{water})}$ is the average counts per min per mL of water and W is the weight of the fish in kg.

An estimate of the theoretical uptake rate of EE2 by drinking alone was made using the following equation:

$$\text{Theoretical uptake rate by drinking} = \text{DR} \times \text{CPM}_{(\text{water})} \times \frac{1}{\text{SA}} \quad (6)$$

where DR is the actual drinking rate that was measured (calculated for a 5-g fish in 16 ppt), $\text{CPM}_{(\text{water})}$ is expressed as the mean radioactivity of EE2 in the exposure water in cpm/mL. SA is the specific activity of EE2 measured from the radioactivity of the stock solution.

Table 1
EE2 uptake rate (ng/5g-fish/h) in recently deceased killifish (*Fundulus heteroclitus*) at 18 °C and 16 ppt. Means ± S.E.M (N = 5).

Organ	EE2 uptake rate (ng/5g-fish/h) in dead fish
Carcass	0.046 ± 0.010
Gut	0.005 ± 0.001
Gill	0.002 ± 0.0006
Liver + gall bladder	0.004 ± 0.020
Spleen	0.0005 ± 0.0001
Total	0.058 ± 0.010

2.9. Statistics

All statistical tests were performed with SigmaPlot 10.0 for linear and non-linear curve fitting and Sigma Stat 3.5 for comparisons of means. Data have been expressed as means ±1 SEM (N = number of fish). For all experimental treatments, N = 5 was used, unless otherwise stated. Simple comparisons of two means were made by Student's unpaired two-tailed t-test. Comparisons among multiple experimental means have been made using a One-Way ANOVA followed by a Tukey's post hoc test, in the case of failed normality, a log transformation was used which was successful in achieving normality. In Figures, values sharing the same letter were not significantly different from one another ($P > 0.05$), whereas values not sharing the same letter were determined to be significantly different ($P < 0.05$). Figure legends denote the specific test performed for each trial.

3. Results

3.1. EE2 exposure levels

Mean exposure levels during the various trials are shown in Fig. 1. In different series, mean concentrations ranged from about 43 to 65 ng/L, and tended to decline slightly over time. Thus, all EE2 uptake rates were adjusted to an exposure concentration of 100 ng/L as outlined in Section 2.

3.2. EE2 uptake rates of dead fish

Experiments with recently deceased killifish under reference conditions (18 °C, 16 ppt) revealed negligible EE2 uptake rates relative to those of live fish, thereby eliminating the possibility that uptake in live fish was by surface adsorption alone. Whole uptake body rates averaged only 0.05 ng/5 g fish/h in dead fish (Table 1), less than 4% of the rate measured in live killifish under the same conditions (Fig. 3A).

3.3. Linearity of uptake experiment

Fish were sampled at 30 min intervals over the course of 2 h. The absolute uptake of EE2 by the whole body increased linearly with time, reflected in a significant positive correlation (r^2 = 0.98, P = 0.0006, Fig. 2A). Therefore flux rates of EE2 were identical when calculated over 30, 60, 90, and 120 min periods (Fig. 2B). With respect to the tissue-specific pattern of EE2 uptake, the carcass accumulated the highest amount of EE2 across all time points ranging from 0.07 ng/5-g fish after 30 min to 0.21 ng/5-g fish after 120 min. Furthermore, the gall bladder was the next highest accumulator, followed by the liver, and gut respectively (Fig. 2C).

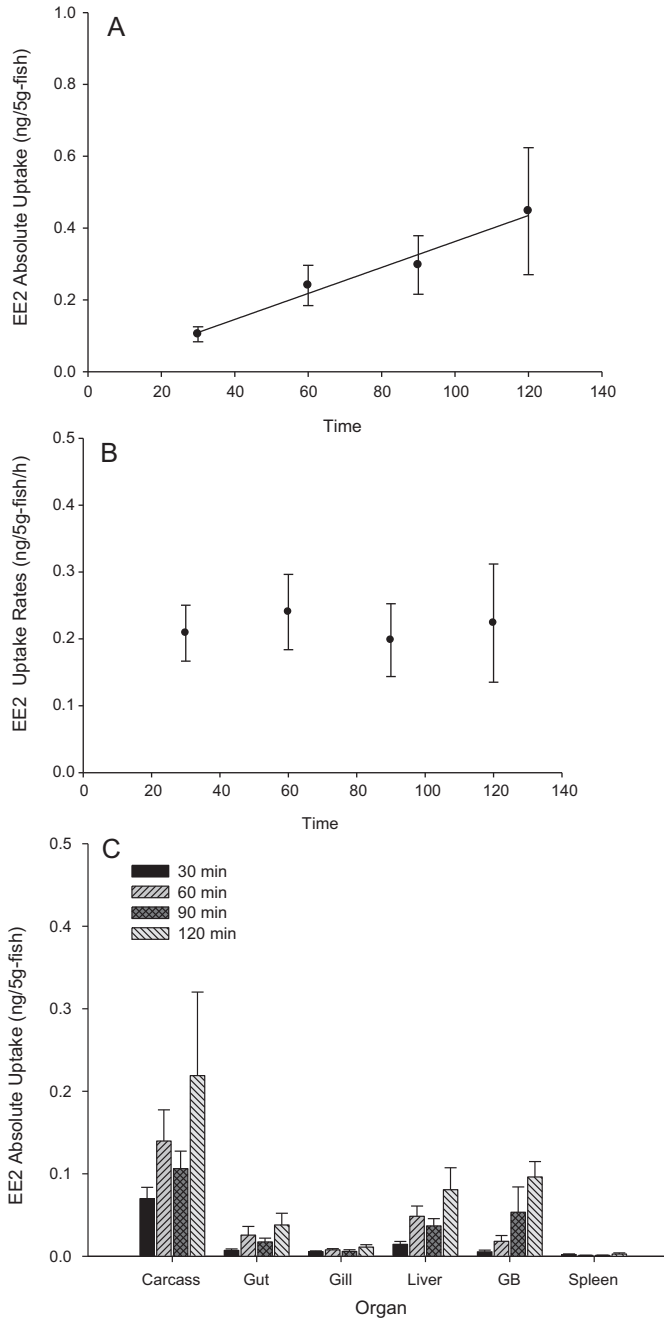

Fig. 2. (A) Absolute uptake of EE2 at specific time points (30, 60, 90, 120 min) normalized to a 5-g fish (ng/5-g fish), in killifish (*Fundulus heteroclitus*) acclimated to 18 °C at a salinity of 16 ppt. Values are means ± S.E.M. (N = 6 per treatment). (B) Uptake rates of EE2 in killifish (*Fundulus heteroclitus*) calculated over specific time periods (30, 60, 90 and 120 min), normalized to a 5-g fish (ng/5-g fish/h), in animals acclimated to 18 °C at a salinity of 16 ppt. Values are means ± S.E.M. (N = 6 per treatment). (C) Absolute uptake of EE2 in specific organs at specific time points (30, 60, 90, 120 min), normalized to a 5-g fish (ng/5-g fish), in killifish (*Fundulus heteroclitus*) acclimated to 18 °C at a salinity of 16 ppt. Values are means ± S.E.M. (N = 6 per treatment). GB = gall bladder.

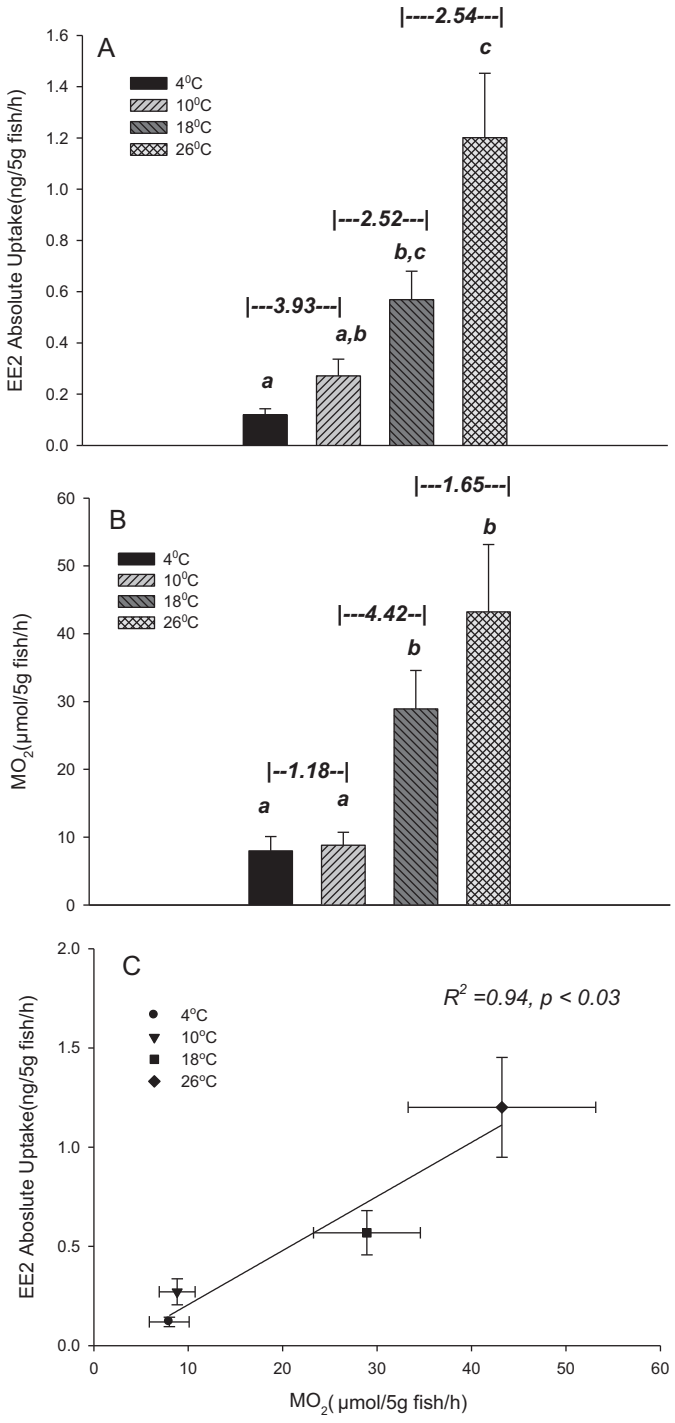

Fig. 3. (A) Whole body EE2 uptake rates (ng/5-g fish/h) in killifish (*Fundulus heteroclitus*) acclimated to 18 °C at a salinity of 16 ppt, then exposed for 24 h to temperatures of 4, 10, 18, and 26 °C at the same salinity. The Q_{10} values for the various temperature intervals are shown. Values are means ± S.E.M. (N = 5 per treatment, except the 18 °C treatment where N = 6 and 4 °C where N = 4). Means with different letters are significantly different (P < 0.05) as determined by a One-Way ANOVA on ranks followed by Tukey's post hoc test. (B) Simultaneous rates of O_2 consumption (MO_2, µmol/5-g fish/h) measured in the same treatments as panel A. The Q_{10} values for the various temperature intervals are shown. Means with different letters are significantly different (P < 0.05) as determined by a One-Way ANOVA followed by Tukey's post hoc test. (C) The overall relationship between whole body EE2 uptake rate and MO_2 (r^2 = 0.94, P < 0.05) at different temperatures in the treatments of panels A and B.

3.4. EE2 and MO_2 at different temperatures

When temperature was decreased from the reference acclimation condition (18 °C, 16 ppt) to either 10 °C or 4 °C over a 24 h period, the whole body uptake rates of EE2 fell markedly from 0.56 to 0.27 and 0.11 ng/5-g fish/h, respectively (Fig. 3A). Uptake rates, however, increased to 1.20 ng/5-g fish/h when temperature was increased to 26 °C (Fig. 3A). A similar pattern was seen in MO_2,

Table 2
Acute (2 h) radiolabeled EE2 uptake expressed as a percentage of total whole body uptake rates in the organs of killifish (*Fundulus heteroclitus*) after 15 day exposure to non-radiolabeled EE2 (chronic) and ethanol (control) at conditions of 18 °C at salinity of 16 ppt. Means ± S.E.M (N = 9).

Organ	Control (ethanol exposed)	Chronic (non-radiolabeled exposed)
Carcass	30.8 ± 5.0	28.6 ± 2.6
Gut	23.9 ± 2.2	22.3 ± 2.9
Gill	4.1 ± 0.3	3.2 ± 0.6
Liver	22.0 ± 2.7	25.7 ± 2.2
Gall bladder	18.3 ± 3.9	19.6 ± 3.7
Spleen	1.0 ± 0.2	0.9 ± 0.3

with significantly lower rates at both 4 °C and 10 °C and intermediate rates at 18 °C and highest rates occurring at 26 °C (Fig. 3B). Q_{10} values for EE2 uptake were similar in both the 10–18 °C ranges and 18–26 °C ranges (2.52, 2.54) with the highest in the 4–10 °C range (3.93), whereas Q_{10} values for MO_2 were high (4.42) only in the 10–18 °C range. For both EE2 uptake and MO_2, Q_{10} values were >1.0 in the 4–10 and 18–26 °C range. Overall, there was a strong positive linear relationship between EE2 uptake and MO_2 (r^2 = 0.94, P < 0.05; Fig. 3C).

3.5. Salinity studies

Changes in salinity had a marked effect on EE2 uptake in killifish. Killifish that had been acclimated to the reference condition (18 °C, 16 ppt) accumulated EE2 at a 3-fold greater rate than freshwater-acclimated killifish, approximately 2-fold greater than both 100% seawater-acclimated animals (32 ppt), and killifish transferred to 32 ppt for 24 h prior. Furthermore, there was nearly 1.5-fold greater uptake rate in the reference condition killifish comparatively to those that had been transferred to fresh water for 24 h prior to exposure (Fig. 4A). Unlike the temperature series (Fig. 3C), there was not a significant positive correlation (r^2 = 0.53, P = 0.165) between EE2 uptake and MO_2 in this series (Fig. 4C).

3.6. Tissue-specific accumulation of EE2 in killifish

Following a 2 h exposure to 100 ng/L of radiolabeled EE2, the tissue-specific pattern of EE2 accumulation was consistent across all temperature and salinity treatments in killifish, and is illustrated by the temperature series in Fig. 5A and B. Highest accumulation always occurred in the liver and gall bladder (sampled together in some of the series). This compartment accounted for at least 40–60% of the total accumulation in all series. When the liver and gall bladder were sampled separately, as illustrated for the killifish in the reference condition (18 °C, 16 ppt), the gall bladder usually accounted for about 35% of the total, while the liver accounted for 25% of the radiolabeled EE2 (Fig. 5B). The carcass accumulated approximately 20–30% of the total burden while the gut usually accounted for 10–20% of the total accumulation. The spleen and gills accounted for <10% of the total.

3.7. Chronic exposure

After a chronic exposure of killifish to non-radiolabeled EE2 (15 days), the rates of EE2 uptake (0.63 ng/5-g fish/h, S.E.M. ± 0.09, N = 9) were unchanged relative to those (0.66 ng/5-g fish/h, S.E.M. ± 0.09, N = 9) in simultaneous control animals exposed to clean water for 15 days. The tissue-specific pattern of uptake also remained unchanged in the organs of both the control killifish and those exposed chronically (Table 2). The carcass was the highest accumulator in both the control and chronically exposed fish, at approximately 30% of the total uptake. The liver was the next highest accumulator in both exposures with the control at 22% and chronic at 25%, followed by the gall bladder at 18% and 19% respectively (Table 2).

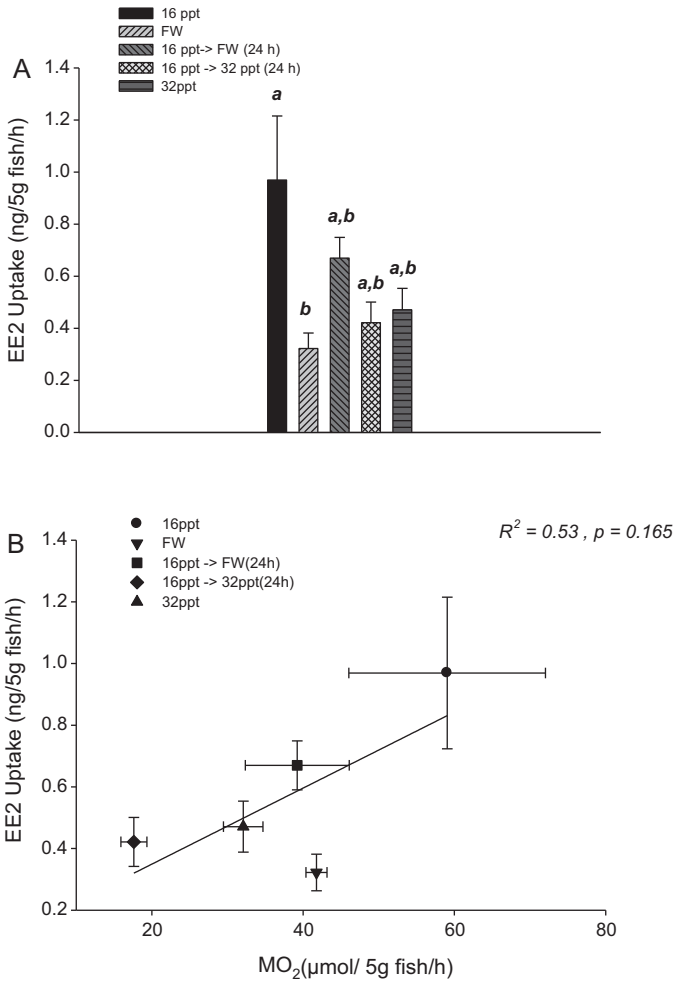

Fig. 4. (A) Whole body EE2 uptake rates (ng/5-g fish/h) of killifish (*Fundulus heteroclitus*) exposed to five different salinity conditions: freshwater acclimation (FW), 16 ppt acclimation (50% sea water), 32 ppt acclimation (100% sea water), 24-h transfer to fresh water after previous acclimation to 16 ppt, or 24-h transfer to 32 ppt after previous acclimation to 16 ppt, all at 18 °C. Values are means ± S.E.M. (N = 4 per treatment, except 32 ppt where N = 10 and 32 ppt transfer series where N = 6). Values not sharing the same letter are significantly different (P < 0.05) as determined by an One-Way ANOVA on ranks followed by a Tukey post hoc test. (B). The overall relationship between the whole body EE2 uptake rates and the simultaneous rates of O_2 consumption (MO_2) measured in the various salinity treatments of panels A and B (r^2 = 0.53, P = 0.165) at different temperatures in the treatments of panels A. Values are means ± S.E.M.

3.8. Drinking

As the gut tissues accounted for 10–20% of the total EE2 accumulation after 2 h (Fig. 6A and B), and absolute EE2 uptake rates were much higher at 16 ppt than in fresh water, we investigated the possibility that drinking accounted for a significant proportion of uptake. Measured drinking rates were about 10 mL/kg/h in killifish acclimated to 16 ppt, approximately 25 times higher than the very low rates (approximately 0.4 mL/kg/h) in freshwater-acclimated animals (Fig. 6A). When the drinking rate at 16 ppt was used to predict the EE2 bioaccumulation seen in the gut (see equation 6, calculation section of Section 2), it accounted for about 30% of the actual measured value (Fig. 6B), or less than 5% of the whole body uptake.

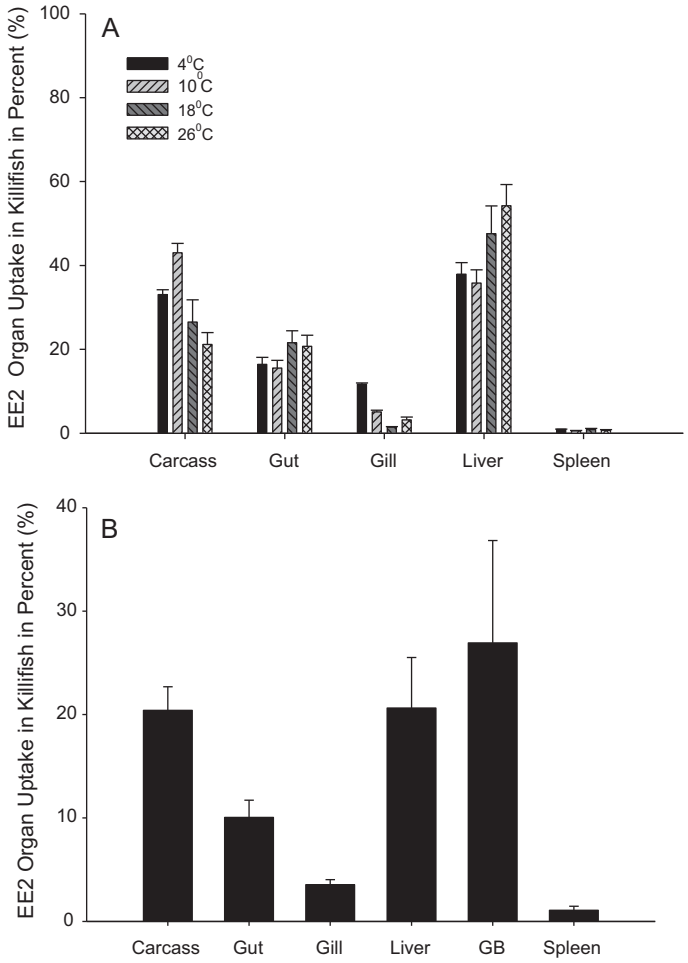

Fig. 5. (A) EE2 uptake rates in specific organs, normalized to a 5-g fish (ng/5-g fish/h), in killifish (*Fundulus heteroclitus*) exposed to temperatures ranging of 4, 10, 18, and 26 °C at a salinity of 16 ppt. Values are means ± S.E.M. (N = 5 per treatment, except the 18 °C and 4 °C treatments where N = 4). Note that the 'liver' contains both the liver and the gallbladder. GB = gall bladder. (B) EE2 uptake expressed as a percentage of total whole body uptake rates in the organs of killifish (*Fundulus heteroclitus*) exposed to 18 °C at salinity of 16 ppt. Note that the gall bladder and liver are measured separately. Means ± 1 S.E.M (N = 4 per treatment).

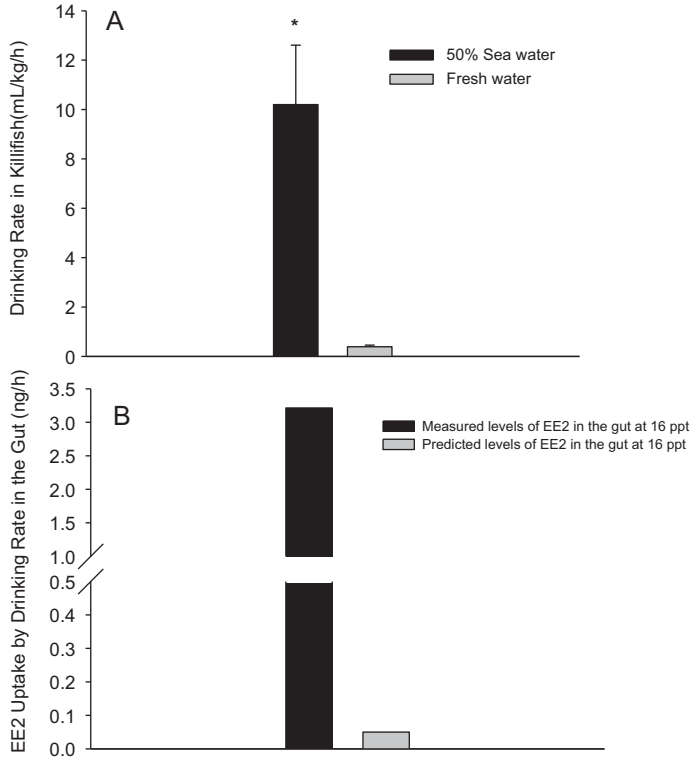

Fig. 6. (A) Drinking rates (mL/kg/h) measured in killifish (*Fundulus heteroclitus*) acclimated to a salinity of 16 ppt or freshwater. Values are means ± S.E.M (N = 5 per treatment). Asterisk indicates significant difference (P < 0.05) by Student's t-test. (B) A comparison, for a 5-g killifish acclimated to a salinity of 16 ppt at 18 °C, of the measured EE2 uptake rate into the gut with the theoretical rate of EE2 uptake that could have been due to drinking of the medium over the same period. See Section 2 for details on calculations.

3.9. Discussion

As hypothesized, we demonstrated that changes in uptake of EE2 occurred under differing environmental conditions. Furthermore, there appears to be a significant relationship between oxygen consumption and EE2 uptake in killifish. We suggest that this is likely due to environmental influences and physiological responses to these influences that affect both oxygen consumption and the uptake of this xenoestrogen.

3.10. EE2 concentrations

The waterborne concentration of EE2 was initially lower than the added dose of 100 ng/L (Fig. 1). Most of the loss was immediate (i.e. prior to 0 min), indicating adsorption of EE2 to the glass walls and plastic lids of the experimental containers, since EE2 is described as "sticky" (Walker and Watson, 2010). The slow continuing losses from 0 min through 120 min likely represented uptake by the fish, as they were in the approximate range of the measured accumulation rates. The substantial decline in the first 60 min of the 32 ppt treatment may have been an artifact of inadequate mixing prior to the 0 min sample.

3.11. Linearity of uptake

This experiment was conducted to provide basis for the assumption that uptake of EE2 follows a linear, proportional pattern with time during acute exposure. This proved to be the case (Fig. 2A), with flux rates remaining constant over the course of the 2 h period (Fig. 2B), thereby validating the approach used in all other trials. The experiment also revealed the very rapid metabolic processing of EE2, with significant appearance of EE2-derived radioactivity in

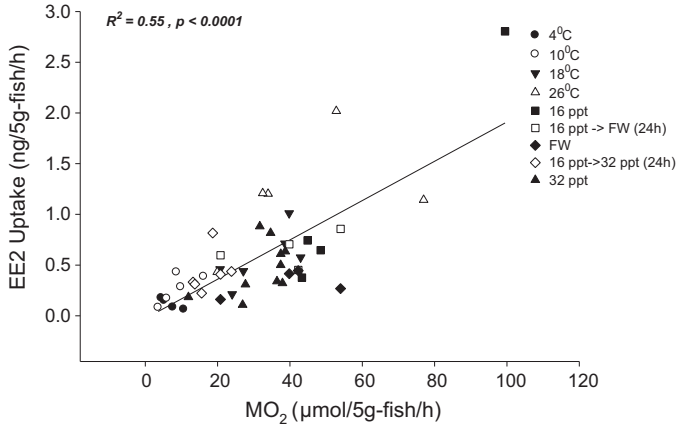

Fig. 7. The overall relationship between the rate of oxygen consumption (MO_2) and the rate of whole body EE2 uptake in individual killifish, with temperatures ranging from 26 °C to 4 °C, and salinities of freshwater, 16 ppt, and 32 ppt in the various experimental series. r^2 = 0.55, P < 0.0001.

the liver and gall bladder after only 30 min of exposure, with steady increases thereafter (Fig. 2C).

3.12. Temperature and EE2

At higher environmental temperatures, the carrying capacity of water for oxygen is decreased, resulting in less dissolved O_2 for any given PO_2. Fish are poikilothermic, so their metabolic rates will tend to parallel water temperature (Hazel and Prosser, 1974). When fish are exposed to increases in temperature, both the volume and rate of buccal pumping (i.e. ventilation) will increase, as will the stroke volume and heart rate (i.e. perfusion) (Randall, 1982). Fish will also enhance lamellar perfusion by increasing arterial blood pressure and dilation of blood vessels, thereby opening a greater proportion of lamellar capillary beds (Booth, 1978; Davis, 1972; Farrell et al., 1980; Hughes and Saunders, 1970; Taylor and Barrett, 1985). The respiratory surface area, therefore, becomes enlarged and the diffusion distance decreased (Nilsson 2007; Yang et al., 2000). While these strategies will enhance oxygen uptake, they may also increase EE2 uptake. Thus, it is not surprising that highest uptake of EE2 occurred at the highest temperature (26 °C, 16 ppt) (Fig. 3A). The opposite is true under cold temperatures; gill epithelial thickness tends to increase as temperature decreases, thereby increasing diffusion distance from water-to-blood (Portner et al., 2004). There was also a great decline in oxygen consumption rates reflecting decreases in cellular metabolic rate, ventilation and perfusion rates, and gill surface area (Nilsson, 2007). These reasons are likely why we see a strong linear relationship between oxygen consumption and EE2 uptake rates as temperature increases or decreases (Fig. 3C).

The Q_{10} value is a commonly-used index that quantifies the rate at which processes change with temperature. If the Q_{10} values are below 1.5, passive processes are predominating, i.e. simple diffusion. However, if the Q_{10} values are above 2.0 then it is likely that the process involves the expenditure of metabolic energy (Kita et al., 1996; Schmidt-Nielsen, 1997). At 4–10 °C, 10–18 °C, and 18–26 °C, EE2 Q_{10} values were above 2.0 indicating that uptake is very dependent on temperature, displaying rates of reactions increasing 2- to 3-fold as temperature increases 10-fold. Notably, O_2 consumption rate also displayed a high Q_{10} over part of this range, so this relationship is not unexpected.

3.13. The mechanism of EE2 uptake

To rule out the possibility that significant EE2 uptake occurred by surface adsorption, an exposure with recently deceased fish was conducted. Uptake by the dead fish was only 4% of the accumulation observed in live fish under the same conditions, and most of the EE2 uptake in the dead fish was absorbed by the carcass, which included the skin (Table 1). Thus, EE2 accumulation in live fish was not by surface adsorption, but rather by a specific uptake and distribution pathway involving the circulatory system, resulting in accumulation in the internal organs of live fish.

Our results indicate that the gills are the likely entry point for EE2 uptake. This is probably because of the lipid-rich composition of the gills and because they are a thin barrier between the aqueous environment and the blood. The diffusion of lipid-soluble compounds across the branchial epithelium has been shown to occur transcellularly through a form of passive diffusion as in the case of oxygen (Brauner et al., 1994; Hunn and Allen, 1974; McKim and Erickson, 1991; Murphy and Murphy, 1971; Satchell, 1984; Yang and Randall, 1995; Yang et al., 2000). We suggest that due to the high octanol-water partition coefficient of EE2 ($\log K_{ow}$ of 4.12) there is an increased likelihood of a high absorption rate across the gill lamellae (Bradbury et al., 1986; McKim et al., 1986, 1987a,b). EE2 diffuses through the gills rather than accumulating in this tissue, and entrance at the gills may be the rate-limiting step in uptake of this organic compound. Our results support this notion as only low levels of EE2 were repeatedly observed in the killifish gills after acute exposures (Fig. 5A and B). Once an organic compound diffuses across the gills it will likely become bound to a protein carrier present in the blood (Schmieder and Henry, 1988). Sex hormone binding globulin (SHBG) has been implicated as the possible transport protein required for EE2 movement in fish (Miguel-Queralt and Hammond, 2008). Interestingly, SHBG is located in the lamellae and has a high affinity for synthetic estrogens (Scott et al., 2005). Thus, if SHBG is the protein carrier that binds EE2, then it is the probable mechanism behind EE2 delivery to specific organs throughout the body.

EE2 uptake in the gut was 10–20% of the total after a 2 h exposure under reference conditions (18 °C, 16 ppt). EE2 could potentially accumulate in the gut not only from the endogenous uptake of EE2 from the circulation, but also because of direct drinking of the external medium, as the gut is the main site of Cl^- and fluid absorption in this species (Scott et al., 2004, 2006). Earlier work indicated that freshwater killifish drink at a rate that is only about 10% that of fish acclimated to brackish water (approximately 14 ppt) (Malvin et al., 1980; Potts and Evans, 1967). Our results are in qualitative accord, revealing that freshwater-adapted killifish drink approximately 25-fold less than those acclimated to 50% sea water (16 ppt) (Fig. 6A). However, the levels of EE2 actually measured in the gut were more than 3-fold higher than predicted if drinking rate were to account for the uptake alone (Fig. 6B), indicating a alternate explanation for the appearance of EE2 in the gut, aside from drinking, as outlined below.

3.14. EE2 accumulation in specific tissues

Together, the gall bladder and liver accounted for more than 50% of total accumulation after 2 h in the reference condition (Fig. 5A and B). However, when these organs were separated, the gall bladder actually accounted for the larger portion, indicating very rapid processing of EE2 into the bile. Liver hepatocytes are known to contain high concentrations of estrogen receptors which will bind EE2, as it is an E2 mimic (Tollefsen et al., 2002; Werner et al., 2003), so it is not surprising that EE2 accumulates preferentially in this organ. It is known that lipophilic xenobiotics, as well as other endogenous and exogenous substances that are found circulating in the blood, are metabolized/transformed by hepatic enzymes in the liver. These substances are then secreted in a water soluble form to the gall bladder where they are incorporated into the bile (Blom et al., 2000; Forlin et al., 1995). In fact, bile sampling is a common method used to detect exposure to xenoestrogens (Fenlon et al., 2010; Ruddock et al., 2003). EE2 in the bile has likely been transformed into phase II metabolites (conjugates of glucuronic acid and sulphate) by the liver. These compounds may be converted back into the parent compound once expelled into the intestine/gut, a process aided by bacterial modifications (Bodzek and Dudziak, 2006; Fenlon et al., 2010). Very rapid biliary secretion of EE2 or its metabolites, as seen in the time course experiment (Fig. 2C) likely provides explanation for the appearance of EE2 in the gut, regardless of drinking rate. However, because of the methodology used in this experiment, we are unable to determine if whole EE2 is appearing in the gall bladder or simply metabolites of EE2.

The carcass accounted for about 30% of EE2 accumulation in killifish, and contains all organs that were not excised; these included brain, kidney, pancreas, testes, bone, muscle and fat deposits. There are high-affinity estrogen receptors found in many tissues including the brain, pituitary, gonads and accessory sex organs and even in bone (Anglade et al., 1994; Bremner et al., 1994; Doncarlos, 1996; Ernst et al., 1991; Komm et al., 1987; Loomis and Thomas, 1999; Smith et al., 1996).

3.15. Chronic exposure to EE2

Both the rates and the patterns of tissue-specific uptake of radiolabeled EE2 over 2 h did not differ between killifish that were chronically exposed to EE2 for 15 days and those that were acutely exposed for only 2 h. This indicates that chronic exposure does not result in an up or down-regulation of the EE2 uptake rate, or in tissue-specific disposition. Furthermore, this is also probably indicative of the fast processing/metabolism of this chemical in the organism and its limited accumulation over the course of 15 days.

3.16. Salinity and EE2

There are major differences in the morphology of the gill epithelia of killifish in fresh water, 50% sea water and 100% sea water. Seawater chloride cells (SWCCs) are either absent or dormant in freshwater killifish, but appear at salinities of 10% (3–4 ppt) or higher; these cells contain invaginations termed apical pores and are found between and below pavement cells (Laurent and Dunel, 1980; Laurent, 1984). Thus, the highest uptake of EE2 observed in 50% salinity was likely due to the apical pores on the outer lamellae decreasing the diffusion distance for EE2. However, as salinity increases, SWCCs become prevalent on the gill epithelium (Wood, 2001) and SWCCs may proliferate onto the respiratory lamellae to deal with the increased ionic load. This movement is essential; however, the abundance and size of these cells will ultimately increase the diffusion distance for gases and oxygen uptake (Perry, 1997, 1998), and presumably EE2 as well. This may explain the lower EE2 uptake rates seen in 100% sea water (32 ppt) versus 50% sea water (16 ppt), and the transfer from 50% sea water to 100% seawater.

The freshwater killifish gill has a unique cell type that may be associated with the fact that they do not actively uptake Cl^- at the gill. These are called cuboidal cells and as a result killifish will not display the typical freshwater chloride cells (FWCCs) present in other teleosts (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003; Wood and Marshall, 1994). These cuboidal cells are thick and intermingled with pavement cells (PVCs), have mitochondria present in them, lack an apical pore, and are in fact cuboidally shaped (Laurent et al., 2006). Furthermore, in fresh water, the SWCCs are few in number and lay dormant, covered by pavement and cuboidal cells, thereby increasing gill epithelial thickness (Evans et al., 2005; Perry, 1997, 1998). Without an apical pore, the diffusion distance becomes increased, resulting in a thickening of the gill epithelia under freshwater conditions, likely accounting for the decreased uptake of both EE2 and oxygen observed in fresh water. In conditions of a transfer from 50% sea water into fresh water for 24 h, there was a greater EE2 uptake rate compared to freshwater-adapted killifish but overall, a lower value than the value in the 50% seawater group. This is likely because over the course of the 24 h there is a rapid proliferation of cuboidal cells, accompanied by destruction and covering of SWCCs, ultimately increasing gill epithelial thickness to adjust for the change in ionic concentration in the water (Laurent et al., 2006).

MacLatchy and colleagues (unpublished data) found a similar trend between vitellogenin (VTG) gene expression and differences in salinity in EE2-exposed fish. At a nominal exposure concentration of 250 ng/L of EE2 at 18 °C, they observed a significantly higher gene expression of both VTG1 and VTG2 in 50% sea water than in fresh water (Nadon, 2010). Our study correlates with this observation, as there was a higher uptake of EE2 at the 50% salinity, which would likely contribute to the higher expression of VTG under similar conditions.

3.17. Relevance of the findings

Our experimental evidence suggests that it may be possible to develop a model to predict the bioaccumulation of organic endocrine disrupting substances as a function of environmental variables. Any environmental condition that alters oxygen demand and consequently oxygen consumption will likely also impact toxicant uptake because oxygen and lipophilic toxicants have similar uptake pathways in the gills. Therefore, the model could be based on the relationship between oxygen consumption and EE2 uptake, reflecting the gill as the entry point of uptake for both lipophilic molecules (Fig. 7). The eventual goal will be to use such a model to predict EE2 uptake under conditions of differing temperature and salinity situations encountered in the estuarine environment, to provide guidance for the location and timing of WWTP discharges. An additional goal will be to incorporate the role of dissolved oxygen variations in such a model.

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