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A species comparison of 17-α-ethynylestradiol uptake and tissue-specific distribution in six teleost fish

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ABSTRACT

Differences exist among fish species in their sensitivity to endocrine disruptors such as 17-α-ethynylestradiol (EE2). We examined whether there were corresponding differences in EE2 uptake rates and short-term internal distribution patterns. Six freshwater species: Japanese ricefish (medaka, *Oryzias latipes*), goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), Atlantic killifish (*Fundulus heteroclitus*) and rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne radiolabelled EE2 (100 ng/L) for 2-h measurements of uptake, tissue accumulation and oxygen consumption rates (MO_2). EE2 uptake rate and MO_2 were relatively consistent among species (2.5–3.0 fold variation), with the only significant differences being a lower EE2 uptake rate in medaka, and lower MO_2 in medaka, goldfish, and zebrafish relative to the other species. EE2 accumulation, however, exhibited two distinct patterns, suggesting differences in metabolic processing. In killifish and medaka, the highest accumulation (~50%) occurred in the liver and gallbladder, whereas in minnow, goldfish, zebrafish and trout, >50% accumulated in the carcass. No significant sex differences were found in killifish or minnow, apart from lower gill tissue EE2 accumulation in minnow females. This study demonstrated that metabolic processing of EE2 may be species-specific and tissue specific EE2 distribution profiles vary. These could be indicative of differences in overall EE2 sensitivity among species.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are capable of modulating and disrupting the structure and function of the endocrine system of animals, consequently producing adverse health effects in the intact organism or progeny by impacting their reproduction (World Health Organization, 2002). The mechanisms of action of EDCs can be divided into: (1) agonistic/antagonistic effects ("hormone mimics"), (2) disruption of production, transport, metabolism or secretion of natural hormones, and (3) disruption of production and/or function of hormone receptors (Goksøyr et al., 2003; Rotchell and Ostrander, 2003). Xenoestrogens in particular can be agonists and/or antagonists of estrogen receptors, and hence can induce several gonadal pathologies in fish (Mills and Chichester, 2005; Denslow and Sepúlveda, 2007).

Ethynylestradiol (EE2) is a potent endocrine disruptor because it mimics the effects of endogenous 17-β-estradiol (E2) (Gomes et al., 2003). It is used in female contraceptive pills and hormone replacement therapies in menopausal women, and enters waterways via wastewater treatment plant effluents (Desbrow et al., 1998; Gomes et al., 2003). In fish, it has been shown to cause altered oogenesis in females, and decreased liver function and intersex in males (decreased testicular growth, production of vitellogenin (Vtg), and early-stage eggs in their testes) (Tyler et al., 1998; Kidd et al., 2007). Many species used in life cycle reproductive toxicity tests, such as fathead minnow (*Pimephales promelas*) (Parrott and Blunt, 2005), zebrafish (*Danio rerio*) (Nash et al., 2004) and Japanese ricefish (medaka; *Oryzias latipes*) (Balch et al., 2004) will reduce or undergo complete shutdown of egg production at EE2 levels of ≤10 ng/L, whereas killifish (*Fundulus heteroclitus*) will continue to produce and fertilize eggs normally, despite exposures to very high concentrations of EE2 (up to 3000 ng/L; Bosker et al., unpublished). In zebrafish, a life cycle exposure to 5 ng/L EE2 caused a 56% reduction in fecundity and complete population failure with no fertilization since males lacked functional testes (Nash et al., 2004). Decreased egg fertilization rates have also been detected in fathead minnow although at much lower concentrations (0.3 ng/L) when exposure covered the period from egg fertilization to adulthood (Parrott and Blunt, 2005).

These reports highlight large apparent differences in EE2 sensitivity among species. Among possible explanations are: (1) there are large differences in EE2 uptake rates among species; (2) there are large differences in the rate at which the compound is transported to various internal organs for action and detoxification; and/or (3) there are physiological differences in receptor specificity and number.

EE2 is a highly lipophilic compound (octanol–water partition coefficient = 4.12), and will likely be taken up by the gills, as they are directly exposed to the surrounding water and their lipid-rich membrane

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facilitates diffusion (McKim and Erickson, 1991; Yamamoto et al., 2003). Oxygen also passively diffuses across the gills so factors that affect oxygen uptake may also influence lipophilic toxicant uptake (McKim and Erickson, 1991; Yang et al., 2000). Not surprisingly then, Blewett et al. (2013a,b) found linear relationships between EE2 uptake and oxygen consumption rates (MO_2) in the euryhaline killifish under several different experimental scenarios. Despite entering through the branchial epithelium, only low levels of EE2 (5% of total accumulations) were observed in killifish gills after 2 h, whereas 40–60% was found in the liver and gallbladder suggesting that EE2 is rapidly metabolized in this species. Most of the remainder was in the carcass (30–40%) with only small amounts in gut and spleen (Blewett et al., 2013a,b).

Since environmental factors (salinity, temperature, oxygen) can have significant effects on EE2 uptake and accumulation patterns in one species (Blewett et al., 2013a,b), it is quite possible that there may be differences in the metabolic processing of this xenobiotic across species. However, despite numerous studies investigating the effects of EE2 on fish development and reproduction, we are not aware of others examining short-term EE2 uptake rates and tissue-specific distribution in fish. Therefore, the present study investigated EE2 uptake at a constant exposure concentration in six teleost fish species with a range of known sensitivities to EE2: medaka, goldfish (*Carassius auratus*), zebrafish, fathead minnow, rainbow trout (*Oncorhynchus mykiss*) and killifish.

We hypothesized that different teleost species would have different uptake rates and/or patterns of EE2 accumulation depending on individual metabolic processing speeds, and that this might correlate with sensitivity to endocrine disruption. Furthermore, we predicted that more closely related species such as goldfish, zebrafish, and fathead minnow (all members of the Order Cypriniformes) would share greater similarities in uptake rates and/or the tissue distribution profile versus less closely related species such as killifish (Order Cyprinodontiformes), medaka (Order Beloniformes) and rainbow trout (Order Salmoniformes). The latter species represents an earlier phylogenetic offshoot, while killifish and Japanese medaka separated later and are more closely related to one another (Steinke et al., 2006). In addition we investigated the effect of sex on uptake rates and accumulation of EE2 in two of the species, killifish and fathead minnow. Previously it has been shown the locus of response differs between sexes, with the livers and ovaries of females, and the testes of males (Tyler et al., 1998) being the major targets of EE2 impact.

2. Methods

2.1. Animal husbandry

Japanese ricefish (medaka; abbreviated as JM on figures) (0.26 ± 0.02 g, from AQUAlity Tropical Fish Wholesale, Mississauga, ON, Canada), goldfish (GF) (2.98 ± 0.49 g, Big Al's Aquarium Supercenter, Woodbridge, ON, Canada), zebrafish (ZF) (0.26 ± 0.03 g, from Petsmart, Ancaster, ON, Canada), fathead minnow (FHM) (males: 3.14 ± 0.32 g; females: 3.95 ± 0.45 g, from Ministry of the Environment, Toronto, ON, Canada) and rainbow trout (RT) (2.00 ± 0.11 g, from Humber Springs Hatchery in Orangeville, ON, Canada) were each housed in 45-L aquaria set up with recirculating, charcoal filtered, dechlorinated tap water from the city of Hamilton (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 $CaCO_3$; temperature = 18–20 °C, DOC = 2.48 mg/L). Adult killifish (KF; males: 3.58 ± 0.22 g; females 4.50 ± 0.30 g), were obtained by seining in June (2012) near Shediac, Bay of Fundy, NB, Canada (46°20′N, 64°40′W). Fish were then transferred to McMaster University (Hamilton, ON, Canada) and held in 400-L aquaria in 10% seawater for at least two months prior to experimentation. Three weeks prior to test, the fish were transferred to 45-L aquaria filled with recirculating, filtered Hamilton dechlorinated tap water (as above). All fish were fed until satiation every day with commercial fish flakes (Big Al's Aquarium Supercenter) and placed under a 12 h light–12 h dark cycle. 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. EE2 uptake experiments

Before EE2 exposure, fish were held in custom-made glass respirometers for 24 h with constant aeration. Respirometer size varied depending on the size of the fish. For both Japanese medaka and zebrafish, 80-mL respirometers were used, and for goldfish, killifish, rainbow trout and fathead minnow, 516-mL respirometers were used. Approximately 24 h prior to experimentation, fish were placed in the respirometers, which were then placed in a re-circulating constant temperature bath maintained at 18 °C. During this time fish were fasted. After 24 h, the respirometer water was gently replaced and the aeration stone was removed. The water was then dosed with 100 ng/L (nominal value) of radiolabeled [^{3}H]17-α-ethynylestradiol, obtained from American Radiolabeled Chemicals (St. Louis, MO, USA) with a specific activity of 7.5 MBq/μg EE2. During exposures, fish were held in airtight respirometers, with each exposure lasting 2 h. Water samples were taken at 0, 60, and 120 min to measure the partial pressure of oxygen (PO_2; 5 mL) and [^{3}H]EE2 radioactivity (1 mL). Once the 2 h exposure period had elapsed, fish were placed into a "cold-displacement" solution of non-radiolabeled EE2 (10 μg/L) for 5 min to remove any radiolabeled EE2 loosely bound to the surface of their body. After 5 min, fish were euthanized with a lethal dose of pH-corrected (NaOH to pH 7) tricaine methanesulfonate (Syndel Laboratories Ltd., Vancouver, BC, Canada). Fish were then dissected and the following tissues were harvested: gills, gut, gall bladder, liver, spleen, gonads (where possible), muscle, brain, and remaining carcass. Brain samples were not taken in trout as this series was performed somewhat earlier.

2.3. Tissue analysis

After harvesting and weighing, the tissues were digested in 1 N trace metal grade nitric acid (Sigma Aldrich, St. Louis, MO, USA), except for the gut which was digested in 2 N trace metal grade nitric acid. The tissue samples were then incubated at 65 °C for 24 h, vortexed, and then incubated at 65 °C for an additional 24 h. Scintillation fluor (Ultima Gold, Perkin Elmer, Waltham, MA, USA) was added. The tissue samples were counted on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer), using the external standard ratio method for quench correction. A tissue digest was used to create a quench curve so that all samples were standardized to a common counting efficiency (the same as that of the water samples).

2.4. Water analysis

Water PO_2 was measured using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) set to the experimental temperature and connected to an AM Systems Polarographic Amplifier (Model 1900, Carlsberg, WA, USA) digital dissolved oxygen meter. For water [^{3}H]EE2 radioactivity analysis, 3 mL of scintillation fluor (Optiphase, Perkin Elmer) was added to the 1-mL water samples, which were then counted using the Tri-Carb 2900TR Liquid Scintillation Analyzer with all values corrected for quench to a common counting efficiency.

2.5. Calculations

Oxygen consumption (MO_2) was calculated using the following equation:

$$MO_2 = \frac{[PO_2]_i - [PO_2]_f}{\Delta t} \times V \times S_c \times Sc$$

where $[PO_2]_i$ and $[PO_2]_f$ are the initial and final partial pressures of oxygen (mm Hg), respectively, Δt represents the time period of the exposure (h), V is the volume of the respirometer (L), S_c is the solubility coefficient (μmol/L/mm Hg), and Sc represents the mass scaling coefficient taken from Clarke and Johnston (1999). Sc was calculated as $10^{0.79\ \log(5/\text{weight})}$, where weight refers to the actual mass of the fish in g. Thus MO_2 was normalized in each trial to represent a 5-g fish. This approach was used to facilitate comparison with previous killifish studies and minimize the influence of allometric effects of body mass on MO_2 values, which were thus expressed as μmol/5 g fish/h.

EE2 uptake rates were calculated by the following equation:

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

where $\text{CPM}_{\text{total}}$ is the total counts per minute of EE2 in the whole body of the fish (cpm), SA is the specific activity of the radioactive stock (cpm/ng), Δt is the total time of the exposure (h), and E is the ratio of the nominal exposure concentration of 100 ng/L of EE2 to the mean EE2 exposure concentration measured at 0, 60 and 120 min for each fish.

EE2 uptake rates were then normalized to a 5 g fish by again using the scaling coefficient provided by Clarke and Johnston (1999) to remove the effect of body mass on uptake rate. This was done as follows:

$$\text{EE2 Absolute Uptake} = \text{EE2 Uptake} \times 10^{0.79\log(5/\text{weight})}.$$

For more detailed methods, please refer to Blewett et al. (2013b).

2.6. Statistics

All linear and non-linear curve fitting were performed with SigmaPlot 10.0 and Sigma Stat 3.5 was used for other statistical analyses. Data have been expressed as means ± 1 SEM (N = number of fish). For all experimental treatments, N = 6 was used, unless otherwise stated. Simple comparisons of two means were made by Student's unpaired two-tailed *t*-tests. Comparisons among multiple experimental means have been made using a One-Way ANOVA followed by Fisher's LSD post hoc tests. In the case of failed normality, log transformations were used which were successful in achieving normality. Arc-sine transformations were used to achieve normality on percentage values. 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. Absolute EE2 uptake rates and MO_2

Whole body uptake rates of EE2 differed about 3-fold among species; rates were greatest in fathead minnow at 3.87 ng/5 g fish/h, followed by goldfish, rainbow trout, killifish, and zebrafish (all not significantly different from one another). Japanese medaka had the lowest uptake rate at 1.32 ng/5 g fish/h which was significantly lower compared to fathead minnow, goldfish, killifish and rainbow trout (Fig. 1A).

Simultaneously-measured MO_2 varied about 2.5-fold among species, with significantly higher values in rainbow trout (49.5 μmol/5 g fish/h) and killifish (42.8 μmol/5 g fish/h) compared to the Japanese medaka (lowest at 20.1 μmol/5 g fish/h), goldfish (22.1 μmol/5 g fish/h), and zebrafish (24.0 μmol/5 g fish/h). Fathead minnow (32.9 μmol/5 g fish/h) displayed intermediate MO_2 values (Fig. 1B).

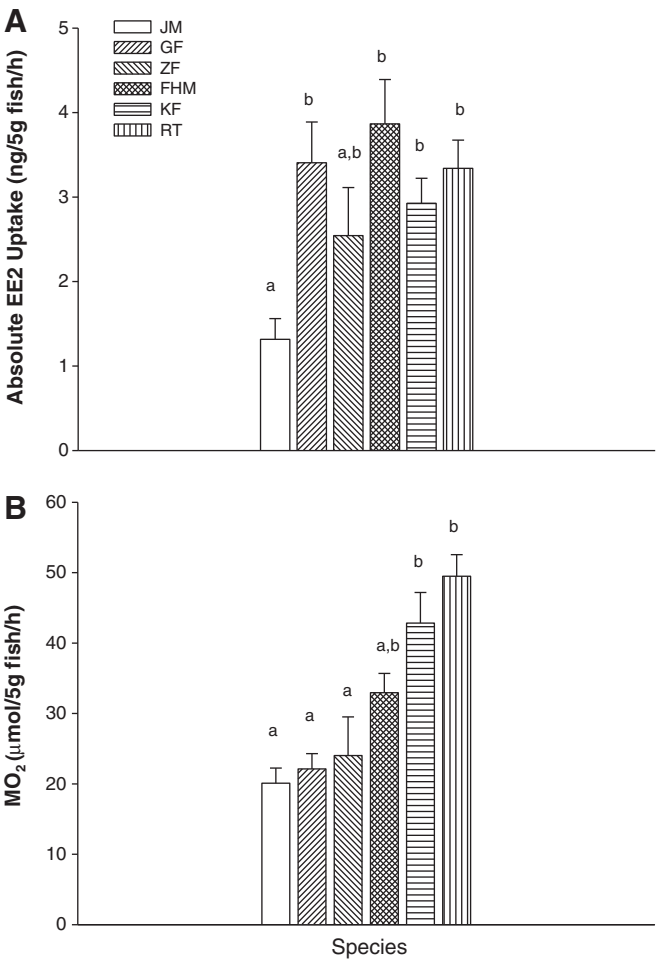

Fig. 1. A. Absolute uptake rate of EE2 in Japanese medaka (JM), goldfish (GF), zebrafish (ZF), rainbow trout (RT), fathead minnow (FHM), and killifish (KF), normalized to a 5 g fish, after 2-h exposure at 18 °C. Values are means ± SEM (N = 6 per treatment, except for zebrafish, where N = 5). Different letters indicate significant differences (one way ANOVA followed by a Fisher LSD post hoc, $p < 0.05$). B. Oxygen consumption in Japanese medaka (JM), goldfish (GF), zebrafish (ZF), rainbow trout (RT), fathead minnow (FHM), and killifish (KF) normalized to a 5 g fish, after 2-h exposure at 18 °C. Different letters denote significant differences (one way ANOVA followed by a Fisher LSD post hoc, $p < 0.05$). Values are means ± SEM (N = 6 per treatment, except for zebrafish, where N = 5).

3.2. Relationships between EE2 uptake and MO_2

Positive linear relationships between EE2 uptake rate and MO_2 were observed for Japanese medaka ($r^2 = 0.75$, $p = 0.03$) and zebrafish ($r^2 = 0.90$, $p = 0.01$) only (Fig. 2A, B). The weak positive relationship for fathead minnow ($r^2 = 0.25$, $p = 0.10$) was not significant (Fig. 2F). Meanwhile, no relationships between EE2 uptake rate and MO_2 were established for either goldfish ($r^2 = 0.21$, $p = 0.35$), killifish ($r^2 = 0.05$, $p = 0.50$) or rainbow trout ($r^2 = 0.0004$, $p = 0.97$) (Fig. 2C, D, E).

3.3. Tissue-specific patterns of EE2 accumulation

There were marked differences in the EE2 tissue distribution patterns among species after only 2 h of EE2 exposure. In Japanese medaka the highest EE2 accumulation occurred in liver and gall bladder, with these tissues accumulating approximately 50% of the total EE2 burden. This was followed by 31% in the carcass, 9% in the gut, 7% in the gill, and approximately 1% each in the spleen and brain (Fig. 3A). For killifish (both male and female), the pattern was very similar, with high liver/gall bladder burden (54%) and low carcass accumulation (~26%) but

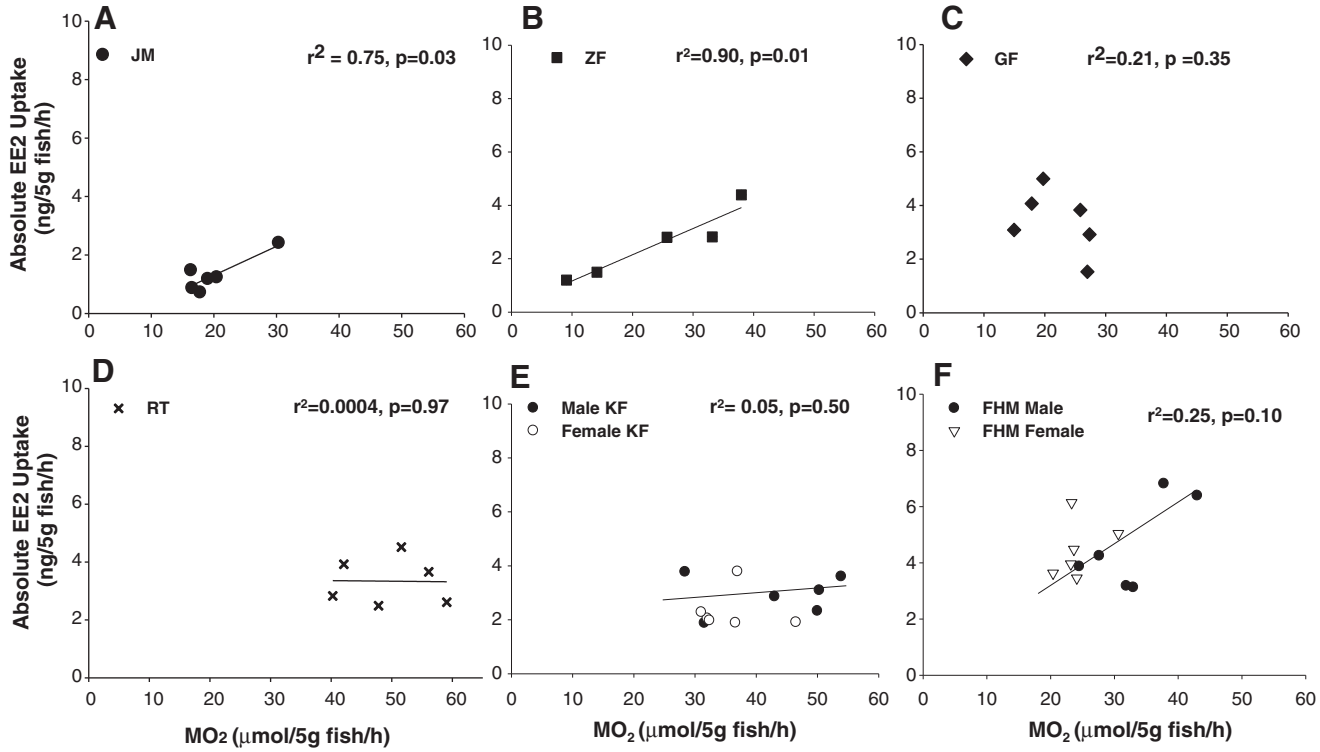

Fig. 2. The overall relationship between whole body EE2 uptake rates and simultaneous rates of oxygen consumption (MO_2) measured in A) Japanese medaka (JM) ($r^2 = 0.75$, $p = 0.03$, B) zebrafish (ZF) ($r^2 = 0.90$, $p = 0.01$) C) goldfish (GF) ($r^2 = 0.21$, $p = 0.35$), D) in rainbow trout ($r^2 = 0.0004$, $p = 0.97$), E) in male and female fathead minnow (FHM) ($r^2 = 0.05$, $p = 0.50$), and F) male and female killifish (KF) ($r^2 = 0.25$, $p = 0.10$). Values are means ± S.E.M. ($N = 6$ per treatment, except for zebrafish, where $N = 5$).

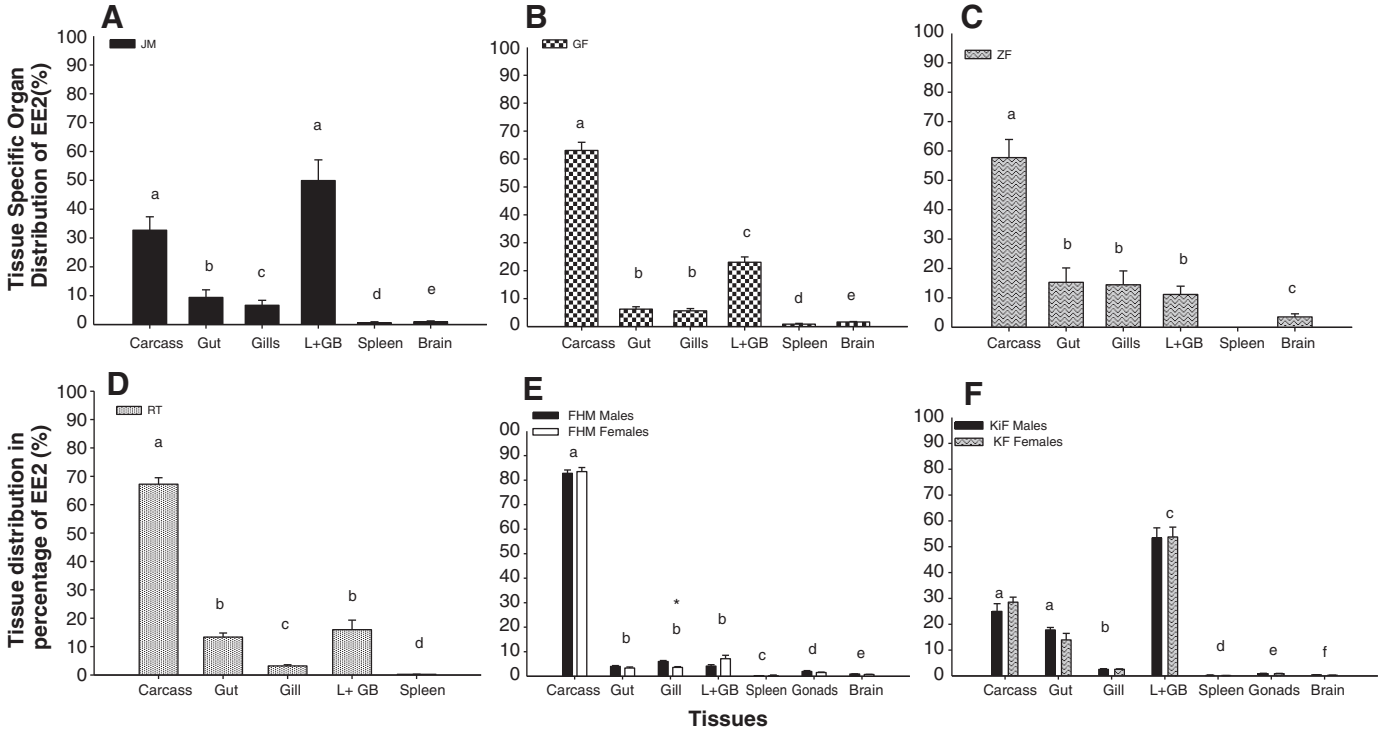

Fig. 3. Tissue specific distribution of EE2 in various tissues after 2-h exposure at 18 °C in A) Japanese medaka (JM), B) goldfish (GF), C) zebrafish (ZF), D) rainbow trout (RT), E) male and female fathead minnow (FHM), F) male and female killifish (KF). Different letters indicate significant differences (One Way ANOVA, $p < 0.05$ followed by a Fisher post hoc test) among tissues. Asterisks indicate significant differences within a tissue (Student's two-tailed *t*-test, $p < 0.05$). Values are means ± SEM ($N = 6$ per treatment except $N = 5$ for zebrafish). L + GB = liver and gall bladder.

slightly higher gut accumulation (~15%) (Fig. 3F). All other killifish tissues were very low in EE2 burden (Fig. 3F). In contrast, the accumulation of EE2 in the carcass was the dominant fraction in goldfish (62%; Fig. 3B), zebrafish (54%; Fig. 3C), rainbow trout (67%; Fig. 3D) and both male and female fathead minnow (84%; Fig. 3E). In goldfish (Fig. 3B), liver and gall bladder were the next most important tissues (23%), while in zebrafish (Fig. 3C), rainbow trout (Fig. 3D), and fathead minnow (Fig. 3E) approximately equal amounts of EE2 distributed to the gut, gills, liver and gall bladder, with a significantly smaller proportion accumulating in the brain (when investigated).

3.4. The influence of sex

Sex could be determined reliably only in killifish and fathead minnow. There were no significant differences in EE2 uptake rates or MO_2 between males and females in either species (Fig. 2E, F). For fathead minnow the only significant difference between the sexes was that the gills accounted for 6% of whole body EE2 uptake in males, but only 3.6% in females (Fig. 3E). Similarly, sex did not significantly alter EE2 distribution in killifish (Fig. 3F).

4. Discussion

Overall, the data confirmed the hypothesis that species would differ in their tissue distribution profiles and suggested that tissue distribution profile could be an indicator of sensitivity to EE2 in fish. However, a surprising result was that absolute EE2 uptake rates remained relatively consistent among species (lower only in Japanese medaka), and between sexes.

4.1. Absolute EE2 uptake among species

Of the species tested, goldfish, killifish, rainbow trout and fathead minnow displayed the highest absolute EE2 uptake rates. There appeared to be a trend in which smaller species (Japanese medaka and zebrafish) had lower uptake rates than larger species (goldfish and fathead minnow) after allometric scaling. However, only Japanese medaka displayed significantly lower uptake rates of EE2 relative to other fish. This consistency was also reflected in the average MO_2 values where medaka again exhibited the lowest rate. This rate was not, however, significantly different from zebrafish, fathead minnow and goldfish, but did differ significantly from the higher rates displayed by trout and killifish. All of the fish were kept in fresh water, tested at the same experimental temperature, and allowed to settle in the same manner, likely ruling out any differences caused by experimental factors. For example, environmental variables such as salinity and temperature change ventilatory volume, gill epithelial thickness and blood perfusion rate, all of which control the transfer rate of oxygen and likely also the uptake rates of lipophilic xenobiotics (Yang et al., 2000). The relative constancy of MO_2 values among fish at rest indicates that under such conditions ventilation, perfusion, and thus diffusibility of oxygen and EE2, are generally similar among species. A similar finding has been reported for a cross-species investigation of water influx at the gill, where three moderately active fish (rainbow trout, yellow perch, and smallmouth bass) were all found to have comparable lamellar surface areas and blood-to-water diffusion distances, and consequently similar water influx rates (McDonald et al., 1991).

4.2. Relationships between absolute EE2 uptake and oxygen consumption rates

EE2 uptake and oxygen consumption were positively and linearly correlated in Japanese medaka and zebrafish. Notably, such significant relationships were not present in goldfish, rainbow trout, fathead minnow, or even killifish. The latter was surprising, as our earlier studies on killifish (Blewett et al., 2013a,b) had found positive relationships between these two variables under some circumstances in this species.

In the present study, our results suggest similar diffusive uptake pathways for EE2 and oxygen in Japanese medaka and zebrafish, as earlier suggested for killifish (Blewett et al., 2013a,b). There appear to be two possible explanations for the absence of such relationships for the other species in the present study. Firstly, given the constant conditions, relatively uniform metabolic rates of the fish, and the confounding effects of inter-individual variability, there may not have been sufficient range of MO_2 to establish such relationships. Another possible confounding factor was our inability to identify the sex of some of the species. In this study, there were significant differences in male and female fathead minnow EE2 gill accumulation (6% in males but 3.6% in females), but no differences in overall EE2 uptake rates. Thus, using a mix of sexes could have uncoupled the linearity of the EE2–MO_2 relationship. In this regard, plasma sex steroid binding proteins (see below) are of particular interest, as these may set the diffusion gradient for EE2 entry. If these proteins vary in concentration among individuals, there again may be uncoupling of the EE2–MO_2 relationships. Other researchers investigating the effects of stress have observed that increased plasma concentrations of cortisol are negatively correlated with concentrations of vitellogenin (Vtg) in female fish (Pottinger and Pickering, 1990). This may be explained by decreases in the number of nuclear estrogen receptor binding sites in hepatic tissues and the relative increase in plasma sex steroid binding proteins (Pottinger and Pickering, 1990). This finding lends some support to the idea that sex could influence the metabolism of endocrine factors and their mimics, and could thus have an impact on the relationship between EE2 and MO_2.

4.3. Species distribution profiles of EE2 accumulation

As previously hypothesized, different species had different tissue distribution profiles of EE2 accumulation. Two main patterns were evident. Japanese medaka and killifish showed high (~50%) accumulation of EE2 in the liver and gall bladder after only 2 h of exposure. Conversely, goldfish, zebrafish, rainbow trout and fathead minnow accumulated the bulk of the EE2 in the carcass. These differences were not correlated with the rate of EE2 uptake, since the medaka had the lowest rate and killifish had a relatively high rate. Furthermore it did not appear to be correlated with body size, as the small medaka and large killifish exhibited one pattern, while the small zebrafish and comparatively larger trout, goldfish, and fathead minnow exhibited the other pattern. These data have three possible explanations, none of which are mutually exclusive: (i) differences in the general physiology and internal metabolic processing (movement of EE2 to different organs); (ii) differences in the distribution of lipid content within the body; and (iii) phylogenetic relationships among these species.

The accumulation of EE2 in different tissues can likely be attributed to the physiology of the fish. The liver metabolizes lipophilic toxicants, which then become incorporated into the bile of the gall bladder for excretion (Forlin et al., 1995; Blom et al., 2000). Differences in EE2 uptake by the liver and gall bladder could reflect differences in rates of metabolism and excretion across species. Our results suggest that the relative rate of metabolic breakdown of EE2 by the liver may be much greater in Japanese medaka and killifish than in other species. To our knowledge, experiments comparing rates of EE2 metabolism among different teleost fish species have yet to be reported. However, a study comparing rat and mouse liver parenchymal cells incubated with EE2 found that despite equivalent covalent binding in the two species, metabolic conversion of EE2 was more efficient in the mouse (Helton et al., 1977). Analysis of the conjugate fractions of the mouse showed mostly glucuronide conjugation, whereas the rat had additional conjugate fractions suggestive of sulfate-conjugation (Helton et al., 1977). If faster metabolism to glucuronide metabolites also applies to teleost fish, then perhaps Japanese medaka and killifish metabolize EE2 into the glucuronide conjugates more readily than the other species, hence explaining

why greatest deposition of EE2 was seen in the liver and gall bladder. Further efforts will be required to determine the conjugates that exist in the gall bladder of fish after an exposure to EE2.

It has previously been reported that sex hormone binding globulin (SHBG) is the transport protein for EE2 (Scott et al., 2005). Recently, it was found that the tissue distribution of SHBG seems to vary among species. In zebrafish, expression of SHBG was found in hepatopancreas and intestine, but in the rainbow trout some expression was observed in the spleen and gill tissue. While in general the liver is the tissue that exhibits expression of SHBG most consistently among different species (Bobe et al., 2010), tissue-dependent expression of SHBG could contribute to the general differences in tissue EE2 accumulation across fish species.

In contrast to killifish and Japanese medaka, the other species (goldfish, zebrafish, rainbow trout and fathead minnow) showed a dominance of EE2 accumulation in the carcass. Differences in lipid body composition vary between fish, and may be a contributing factor to the observed patterns. EE2 is strongly lipophilic; thus it readily associates with fat-soluble compounds including lipids themselves. Indeed, a study performed on shorthead redhorse suckers (*Moxostoma macrolepidotum*) showed that EE2 concentrations were correlated with total body lipid content (Al-Ansari et al., 2010). For most of the fish species we studied, the lipid content and distribution among organs has not been systematically investigated (Henderson and Tocher, 1987), though these characteristics have been reported to vary with the reproductive cycle and season (Jørgensen et al., 1997; Ekman et al., 2009; Rasheed, 2011). Such differences within and between species could significantly impact the accumulation profiles of EE2, and may explain the different patterns observed in the present study.

A final possibility explaining differences in accumulation patterns is phylogeny. Goldfish, zebrafish and fathead minnow all belong to the Order Cypriniformes, while Japanese medaka and killifish belong to the Superorder Acanthopterygii (Order Cyprinodontiformes for killifish and Order Beloniformes for Japanese medaka; Matsumoto et al., 2006; Steinke et al., 2006; Dorts et al., 2009). Rainbow trout (Order Salmoniformes) represent a more primitive species. The similarity in rainbow trout pattern to that of the Cypriniformes order, suggests that this is a phylogenetically more conserved pattern of metabolic processing of estrogens, while the faster distribution to the gall bladder and liver in medaka and killifish may represent a more advanced pattern. Furthermore, the estrogen receptor (which binds to and mediates the effects of EE2) varies among fish species. For example, killifish estrogen receptors were more similar to those of the Japanese medaka and the mangrove killifish (*Krytolebias marmoratus*), than to the estrogen receptor of both the goldfish and rainbow trout (Orlando et al., 2006).

4.4. Sex differences in EE2 uptake and MO_2 in fathead minnows and killifish

Whole body EE2 uptake and most tissue-specific EE2 uptake rates were not significantly different between male and female fathead minnow or killifish. This result was surprising as the effects of exogenous estrogen exposures are usually different for males and females due to differences in the number and distribution of hormone receptors (Tyler et al., 1998; Ekman et al., 2009). Such exposures impact the synthesis of Vtg and other aspects of lipid synthesis and transport, with different effects observed between the sexes since these processes are controlled by circulating steroid levels (Ekman et al., 2009). Ekman et al. (2009) found sex-dependent and temporal alterations in hepatic lipid profiles, total cholesterol, and plasma Vtg levels in fathead minnow exposed to 100 ng/L EE2. Notably in our study over 80% of the EE2 was sequestered in the carcass of both male and female fathead minnow after 2 h, rather than in the liver and gall bladder which accounted for less than 7% of the total. Together, these findings suggest that our 2-h exposure may have been too short to discern sex differences in EE2 distribution since previous demonstrations of sex differences in fathead minnow appeared during longer EE2 exposures (Ekman et al., 2009).

Interestingly, the only organ to display a difference between the sexes was the gill with overall lower accumulation occurring in the female relative to male fathead minnow. The exact mechanism for this difference is unknown, but again may relate to SHBG levels. It is known that SHBG is a significant determinant of lipophilic xenobiotic uptake across the fish gill (Miguel-Queralt and Hammond, 2008). It is further known that levels of SHBG fluctuate over time, and that these fluctuations differ in their extent and timing in male and female sea bass (Miguel-Queralt et al., 2007). If a similar pattern occurs in fathead minnow then this could alter the flux of EE2 through the gills, and/or the concentrations of EE2 that may be present in plasma that is trapped in the gill tissue. These differences could therefore explain sex-dependent gill EE2 burdens. Similarly, a lack of difference in other potential EE2 ligands found in fish tissue could explain the relative lack of other sex-dependent differences in accumulation. For example, Kloas et al. (2000) found the presence of a single class of estrogen binding sites with no significant difference between dissociation constant values in male versus female carp. If similar affinities exist for hepatic EE2 ligands in males and females in the studied species, this may explain why sex differences in liver and gall bladder accumulation were not evident in our study.

It is also important to note that not all females in this study were necessarily at the same stage of oogenesis. Consequently, differences in female EE2 uptake patterns, both within and between species, could have been influenced by factors such as altered VTG gene and protein levels, and SHBG titres.

4.5. Differences in processing and its relationship to sensitivity

There were clear differences in EE2 processing between species. If this is indicative of different sensitivities to EE2 exposure, it could therefore be hypothesized that species displaying a more rapid metabolic processing (i.e. higher levels of EE2 in metabolically active tissue such as the liver) would show higher tolerance to EE2 (i.e. decreased incidence of intersex and greater reproductive success). Species such as the fathead minnow (Parrott and Blunt, 2005) and zebrafish (Nash et al., 2004) display altered reproductive function at levels lower than 10 ng/L, categorizing them as sensitive species. Indeed, both zebrafish and fathead minnow show induction of VTG, ovatestes (100% in a full life cycle exposure; Fenske et al., 2005), and decreased time to sexual maturity, at exposures less than 5 ng/L (Laenge et al., 2001; Nash et al., 2004; Schaefers et al., 2007).

These two species also share a similar tissue distribution pattern of EE2, suggesting that this could be a factor influencing sensitivity. In contrast to fathead minnow and zebrafish, EE2-exposed killifish display relatively unaltered reproduction at such low EE2 concentrations. Even though plasma Vtg concentrations increase (MacLatchy et al., 2003) and plasma steroid levels are altered (MacLatchy et al., 2003; Hogan et al., 2010), they will continue to produce and fertilize eggs normally. This occurs even if they have been exposed to very high concentrations of EE2 (3000 ng/L; Bosker et al., unpubl. observations). The present study shows that this relative tolerance is accompanied by an EE2 tissue distribution profile distinct from that of zebrafish and fathead minnow, in that most EE2 was found in the liver and gall bladder. This suggests mobilization of EE2 to the liver for rapid metabolic processing, and hints that this could be a mechanism responsible for the higher tolerance of killifish.

Interestingly Japanese medaka show a similar EE2 tissue distribution pattern to killifish, with high levels in the liver and gall bladder. If the hypothesis regarding tissue distribution and sensitivity is correct then it would be predicted that Japanese medaka would also be relatively tolerant to EE2 exposure. Indeed, after a 21 day exposure Japanese medaka only displayed an induction of VTG and ovatestes at an EE2 level of 63.9 ng/L, a value almost 15 fold higher than that at which zebrafish display similar impacts. Only at the highest concentration of 488 ng/L was fecundity lost (Seki et al., 2002; Ma et al., 2005; Kashiwada et al.,

2007). Another study by Tilton et al. (2005) shows that after 14 days of exposure to various levels of EE2, it was only at relatively high levels of 500 ng/L that significant reproductive impacts were observed in medaka.

These studies would support the idea that rapid accumulation in the liver, presumably leading to rapid metabolism of EE2, confers higher tolerance to a fish species, at least over short exposure times. However, the contrasting data among different studies as to EE2 tolerance, suggests that more research is required to confirm a relationship between sensitivity and EE2 processing. It is important to note, however, that there are two general differences with our study relative to previous published literature. Our fish were exposed to EE2 for only 2 h, thus the kinetics displayed in this paper may be altered after a long-term exposure. However, at least for killifish, after a 15-day exposure to EE2 at 100 ng/L the pattern of uptake was very similar to that reported in the current study (Blewett et al., 2013a). Furthermore, it is important to note that our study used a relatively high concentration of EE2 (100 ng/L), higher than that used in previous studies on zebrafish and fathead minnow, and this may also have an influence on metabolic processing.

5. Conclusion

The results from our investigation suggest that absolute whole body EE2 accumulation rates and tissue-specific patterns of EE2 accumulation vary among teleost species. These findings suggest the need for future studies on metabolic pathways of EE2 processing and breakdown in different species of fish. If metabolic processing can be related to species sensitivity it may be a possible way to identify species that have the greatest difficulty metabolizing EE2 and as a result will suffer the greatest negative effects upon environmental EE2 exposure.

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