

**INVESTIGATING THE ENVIRONMENTAL INFLUENCES ON THE
ACCUMULATION OF ETHYNYLESTRADIOL IN MARINE TELEOSTS**

**ENVIRONMENTAL INFLUENCES ON THE ACCUMULATION OF
ETHYNYLESTRADIOL IN MARINE TELEOSTS**

By

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ABSTRACT

The synthetic estrogen 17 α -ethynylestradiol (EE2), an endocrine disruptor originating from birth control and hormone replacement therapy, is discharged in wastewater treatment plant (WWTP) effluents. The present study employed radio-labeled EE2 to examine the impact of temperature and salinity on the uptake of EE2 in male killifish (*Fundulus heteroclitus*), a model euryhaline teleost. Fish were exposed to a nominal concentration of 100 ng/L EE2 for 2 h. Actual concentrations were lower due to EE2 adsorption to the exposure system, but uptake rates were normalized to 100 ng/L. Oxygen consumption rates (MO₂), whole body EE2 uptake rates, and tissue-specific EE2 distribution were monitored. EE2 uptake by freshly killed fish was negligible. In killifish acclimated to 18°C at 16 ppt (50 % seawater), MO₂ and EE2 uptake were both much lower after 24-h exposure to 10°C and 4°C, and increased after 24-h exposure to 26°C. Transfer of killifish to fresh water for 24 h tended to lower EE2 uptake rate, and long-term acclimation to fresh water reduced it by about 70 %. Long-term acclimation to 100 % sea water (32 ppt) also reduced EE2 uptake rate by about 50 % relative to 16 ppt. However this was not seen in juvenile rainbow trout (*Oncorhynchus mykiss*) where uptake rates were the same in FW- and 16 ppt-acclimated trout. The tissue-specific accumulation of EE2 was found to be the highest (40-60 % of the total) in the liver plus gall bladder across all exposures, and the great majority of this was in the bile in killifish, regardless of temperature or salinity, whereas in trout accumulation was the highest in the carcass at 70 % of the total. The carcass was the next highest accumulator (30-40 %) in

killifish, followed by the gut (10-20 %) with only small amounts in gills and spleen. Drinking rate, measured with radio-labeled polyethylene glycol-4000, was about 25-times greater in 16 ppt-acclimated killifish relative to freshwater-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 pathways for uptake across the gills of these lipophilic molecules. These data will be useful in developing a predictive model of how variations in key environmental parameters (salinity, temperature, dissolved oxygen) affect EE2 uptake in estuarine fish, so as to determine optimal timing and location of WWTP discharges.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee. It consists of three chapters. Chapter one is a general introduction and overview of background material and the objectives of work. Chapter two consists of a discrete manuscript that is in preparation for submission to a peer-reviewed scientific journal. Lastly, chapter three summarizes the major findings of this thesis, places these findings in the context of current knowledge, and indicates future directions that this research might take.

Chapter 1: **General introduction and thesis objectives**

Chapter 2: **Environmental influences on 17- α ethynylestradiol uptake and the relationship to oxygen consumption in the model euryhaline teleost (*Fundulus heteroclitus*)**

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Chapter 3: **Summary of findings and conclusions**

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ABBREVIATIONS

EDS – Endocrine Disrupting Substances

FW – Fresh Water

SW – Sea Water/Salt Water

E2 – Estradiol

EE2 – Ethynylestradiol

SHBG – Sex Hormone Binding Globulin

VTG – Vitellogenin

SWCC – Saltwater Chloride Cell

FWCC – Freshwater Chloride Cell

PVC – Pavement Cell

PO₂ - Partial Pressure of Oxygen

MO₂ - Oxygen Consumption

CHAPTER 1: GENERAL INTRODUCTION

The endocrine system is a fundamental contributor to the survival of any vertebrate. This system utilizes hormones as “chemical messengers” to exert control over metabolic processes, growth and development, and reproduction (Colburn *et al.*, 1993). Because this system is so essential, any alteration of hormone delivery or uptake into the tissues can have adverse and far-reaching effects (Goldman and Koduru, 2000). More specifically, fish and other vertebrates require exact timed delivery and uptake of hormones over the course of their life cycles, to maintain function. For several decades, increased use of pharmaceuticals and synthetic chemicals has led to increased environmental exposure. Of these pharmaceuticals, among the most damaging are those that impact the endocrine system and are thus termed endocrine disruptors (Tyler *et al.*, 1998; Goldman and Koduru, 2000). These endocrine disruptors can alter normal hormonal function such as hormone synthesis, catabolism, signal transduction, and both secretion and transport of normal hormones (Tyler *et al.*, 1998). Fish exposed to these chemicals at any point in their life cycle may have altered physiological functions of several physiological systems (Tyler *et al.*, 1998).

Once in the aqueous environment these endocrine disruptors will partition and often bioaccumulate in aquatic vertebrates (Tyler *et al.*, 1998). Factors affecting the uptake of these chemicals include the physico-chemical properties of the aquatic environment, the exposure concentration of the chemical, and the fish’s response to the ever-changing external environment. However, the hypothesis that environmental

variables affect the rate of uptake of endocrine disruptors has yet to be extensively tested. Therefore a study that simulates real-world situations by exposing a widely euryhaline thermo-tolerant fish to environmental changes in temperature and salinity, to determine how this will affect the rate of uptake of a well-known endocrine disruptor, is warranted.

Endocrine Disruptors

Anthropogenic pollutants and chemicals have been entering aquatic systems for decades (Colborn *et al.*, 1993). Approximately 100,000 anthropogenic chemicals are in circulation, some of which end up within the aquatic environment (Hartung and Rovida, 2009). Once in the environment, these chemicals interact with one another while degrading at varying rates, and sometimes form highly complex mixtures to produce new chemicals (Sumpter, 1998). Although there are regulations preventing the discharge of pollutants, many are not effectively removed through water treatment procedures and as a result freely enter the environment. Some of these chemicals specifically affect the endocrine system of organisms, and thus have been given the name of endocrine disrupting substances (EDS's). Most of these EDS's are used in major industries, such as the petrochemical industry, soap and detergent industries, and the pharmaceutical industry; they occur in such forms as organopesticides, dioxins, and synthetic estrogens (Jobling and Sumpter, 1993). More specifically, some of these chemicals are called hormone mimics; they alter reproductive function, general survival and overall fecundity by competing with normal hormone actions (Sumpter, 1998; Peters *et al.*, 2007).

Hormone mimics have extensive effects on physiological systems such as triggering hormone secretion, hormone synthesis and interactions with the hypothalamic-pituitary-gonadal axis (Tyler *et al.*, 1998). Of the many endocrine disruptors present in the environment, some that induce the strongest effect are the synthetic estrogens, having 10 to 50 times more potency than endogenous estrogens *in vivo* (Segner *et al.*, 2003). Perhaps the most potent is 17- α -ethynylestradiol (EE2), the synthetic hormone found in the birth control pill which enters the aquatic environment via feces and urine in sewage outflows (Ternes *et al.*, 1999; Thorpe *et al.*, 2003; Peters *et al.*, 2007). The concentrations of EE2 present in aquatic environments are variable depending on country. In Canada, levels as high as 47 ng/L have been reported (Peters *et al.*, 2007) and in the USA, values as high as 273 ng/L have been detected (Kolpin *et al.*, 2002) in sewage treatment plants. Normal sewage outflows, however, are usually found to contain around 1-10ng/L EE2 in both Canada and USA (Peters *et al.*, 2007; Ternes *et al.*, 1999), though it has been noted that as little as 0.1 ng/L of EE2 can induce adverse effects in aquatic organisms (Purdum *et al.*, 2004).

Ethinylestradiol

Ethinylestradiol (EE2) is a synthetic estrogen used in the female contraceptive pill and hormone replacement therapy (Ternes *et al.*, 1999; Thorpe *et al.*, 2003; Peters *et al.*, 2007). Human females metabolize EE2 in the liver, resulting in two separate conjugates, namely glucuronic acid and sulphate. This pathway leaves EE2 inactive within the human body, and these ensuing conjugates are excreted with bile (Abdel-Aziz *et al.*, 1970). However, due to the hydrophobic nature of EE2 (a log K_{ow} of 4.12, which indicates a high lipophilic tendency), the conjugates actually become bound together again to create EE2 in sewage effluents, returning it to a biologically active state (Peters *et al.*, 2007; Tyler *et al.*, 1998). In addition, this lipophilicity means that the chemical can bioaccumulate as much as 100,000 fold in aquatic organisms (Tyler and Sumpter, 1996).

In mammalian systems, and to a lesser extent in fish, there is a general understanding of how this chemical is metabolized and degraded (see Tyler *et al.*, 1998). However, it appears that there are extreme differences between phylogenies in terms of detoxifying mechanisms. In fact, studies to date suggest that teleosts do not display a deactivating system similar to that of humans (Langston *et al.*, 2005). Steroid metabolism is directly related to cholesterol degradation by a series of hepatic cytochrome P450 members (CYP enzymes) through the process of oxidative metabolism (Bjoerkhem, 2002; Zhu and Conney, 1998). They participate in the synthesis of lipids but are the main deactivating mechanism of foreign compounds (Goldstone *et al.*, 2007). Mammalian enzymes are crucial for the metabolism of 17 β -estradiol (E2), specifically the subfamilies of CYP1A,

CYP1B and CYP3A (Scornaienchi *et al.*, 2010). However, there are distinct differences in the CYP1 and CYP3 families across all vertebrates, making it difficult to relate mammals to fish. For example, mammals contain multiple genes of the CYP3A family (humans have four); however, fish only contain one or two depending on the species of the CYP3A's (Qiu *et al.*, 2008). Thus, it is very difficult to draw any sort of conclusions about deactivating mechanisms from mammals to fish considering the large differences in enzyme expression.

The mechanism of action of EE2 is related to both its structure and its chemical properties. In teleosts, EE2 mimics the effects of the natural estrogen, E2 because both steroids contain a phenanthrene ring (Fig. 1.1), and are lipophilic in nature. E2 is important for several processes involved in sexual development of teleosts, from dictating fertility to controlling secondary sexual characteristics in females (Tyler *et al.*, 1998). E2 also plays a role in oviparous teleosts, regulating the production of the phospholipoprotein vitellogenin (VTG) in the liver of fish. VTG is an egg yolk precursor protein that is important for proper formation of the developing oocytes (Jobling *et al.*, 1995a). Indeed, E2 is vital for egg formation, oocyte growth and development, and proper yolk constituents within the oocyte (Tyler *et al.*, 1996). Furthermore, E2 plays a key role in sexual differentiation during development. The female phenotype in fish develops in the presence of E2 whereas the male phenotype develops in its absence (Hunter and Donaldson, 1983; Donaldson, 1996).

When fish are exposed to EE2, this compound competes with E2 for binding sites at estrogen receptors. Once bound to a nuclear or cytoplasmic receptor, the synthetic estrogen will cause a cascade of inducible effects, as if E2 has been bound. For instance, EE2 will induce the effects seen by normal estrogens by initiating a sequence of estrogen signaling pathways, followed by translation of one or more specific proteins (e.g, VTG), even when these pathways might be dormant normally. This is particularly harmful to any organism, male or female, as it will induce the estrogen pathway regardless of normal endocrine function or sexual maturity (Tyler *et al.*, 1998).

Vitellogenin Synthesis and Adverse Effects of EE2

The vitellogenic gene and thus the expression of VTG is under multi-hormonal control, however, E2 plays the strongest role in the synthesis of VTG (Carnevali *et al.*, 1992; Carnevali *et al.*, 1993; Carragher *et al.*, 1989; Rabelo and Tata, 1993). Synthesis of VTG occurs in the liver of most vertebrates and VTG is accumulated by growing oocytes in female fish, where it ultimately ends up as a fuel source for developing embryos (Sumpter and Jobling, 1995). When female fish are in a sexually-maturing state, normal concentrations of VTG rise steadily, concurrent to increasing E2 concentrations (Tyler *et al.*, 1998). Large amounts of VTG are needed for females to provision vast amounts of eggs in the ovary, which can comprise up to 25% of body weight in female fish (Sumpter and Jobling, 1995). In male fish, however, very little VTG can be detected under normal conditions, likely because estrogen concentrations in male fish are too low

to trigger any expression of the gene, even though the gene is present (Chen, 1983). This makes VTG an excellent biomarker for exposure to synthetic estrogens, because its expression in male fish would suggest that estrogenic pathways are becoming activated (MacLatchy *et al.*, 2003). In the presence of these synthetic estrogens, male fish can show induction of VTG as high as 100 mg/ml in plasma, levels comparable to or higher than those in mature female fish (Purdom *et al.*, 1994; Tyler *et al.*, 1996; Tyler *et al.*, 1998).

With exposure to EE2, the large induction of VTG can effectively alter normal egg production in females, but can also cause kidney and liver damage in male fish (Howell *et al.*, 1989). Furthermore, the adherence of EE2 to estrogen receptors in male fish can cause a battery of inducible effects, such as intersex, which is the development of ovarian processes in the tissue of the testes. This is achieved by decreasing circulating hormone levels of 11-ketotestosterone and testosterone, and by inhibiting testis growth (Peters *et al.*, 2007). The appearance of VTG and intersex are usually partnered, and are clear indicators of an altered hormonal state in males. As mentioned earlier, low concentrations (< 2ng/L) of EE2 can cause a wide variety of inducible effects. These levels have been shown to slow development and growth in juvenile rainbow trout (*Oncorhynchus mykiss*) by 50% (Jobling *et al.*, 1995b), and as little as 0.1ng/L of EE2 causes rapid induction of VTG in both male and female fish (Purdom *et al.*, 1994). Furthermore, exposure to 10ng/L of EE2 for four weeks causes reduction in the number of eggs oviposited in fathead minnow (*Pimephales promelas*) (Schweinfurth *et al.*, 1996). With adverse effects like these, there is a possible risk of population collapse in fish due

to chronic EE2 exposure. Research performed by Kidd and colleagues (2007) in a natural lake found that a 3-year exposure to EE2 (whole-lake study, mean exposure of 5-6 ng/L) caused intersex in male fathead minnows, induction of VTG in both sexes, and extended VTG production in females past the breeding season, leading to an overall population collapse of fathead minnow in the test lake.

Thus, all stages of fish development are very sensitive to hormonal alteration. Timed release and concentrations of natural estrogens in the blood stream are carefully managed by the endocrine system. If synthetic hormones accumulate at any point in the life cycle of fish there may be adverse effects. Therefore, a clear understanding of the surrounding environment and concentrations of the chemical become very relevant in determining if the estrogen mimic will bioaccumulate and bioconcentrate in natural aquatic systems (Tyler *et al.*, 1998). Furthermore, delineating the route of uptake into the organisms is key for understanding the possible induction of toxic effects. Indeed, while there has been a vast amount of research on the impacts of EE2 and other EDS's on fish, there has been surprisingly little work on the mechanism(s) by which these substances are taken up, or on how environmental variables influence the rate of uptake. These issues are the focus of my thesis.

Gill Filament Anatomy

In the aquatic environment, the most likely site of uptake of any waterborne xenobiotic is the gills. The gills are composed of a relatively thin barrier (no more than a

few microns) between the outer environment and the blood of the organism that is directly exposed to the aquatic environment (Wood, 2001). In teleost fish, water enters through the mouth, travels into the pharynx where it passes over the gill filaments, flows by the inner wall of the operculum and exits via the posterior opening located in the operculum, a process called buccal pumping (Evans *et al.*, 2005). There are four gill arches on each side of the body, each displaying two rows of filaments. Water travels over the filaments between projections known as lamellae. The individual lamellae are composed of two epithelial sheets held apart by pillar cells, and the blood flows in the spaces around these cells (Evans *et al.*, 2005). The gill epithelium, which is the barrier between the external environment and the extracellular fluids, primarily consists of pavement cells (PVCs) and mitochondrial rich cells (MRC's), comprising 90 % and 10 % of the total gill cells, respectively (Laurent, 1984; Wilson and Laurent, 2002). PVCs are the main site of gas exchange, whereas in general MRC's are the sites of ionoregulation in differing salinities (Evans *et al.*, 2005).

Relative to the ventilation of lungs with air, fish gills are hyperventilated with water, because the oxygen content in water is much lower per unit volume than in air, or in blood (Cameron and Davis, 1970). During the ventilatory process, water flows over the lamellae as blood flows simultaneously in the opposite direction. This creates a counter-current exchange allowing for the maintenance of a high diffusion gradient for oxygen and as well as exogenous substances (Steen and Krusysse, 1964). Oxygen diffuses across the gill lamellae transcellularly, due to its small molecular weight and lipid solubility (Yang *et al.*, 2000). Thus it is logical that a lipid soluble xenohormone (e.g., EE2) could

also be taken in via this pathway. Indeed, anything that might impact oxygen transfer and uptake across the gills might therefore similarly affect xenohormone uptake. Variables such as ventilation volume, gill epithelial thickness, and perfusion all control the transfer rate of oxygen and, potentially, of toxicants, across the gill epithelium; any sort of environmental stress which places demands on the gills could alter these variables (Yang *et al.*, 2000).

The Route of Uptake of EE2

It has been well documented that xenobiotic compounds likely partition and enter across the gill epithelium (Murphy and Murphy, 1971, Satchell, 1984). The gills are ideal as exchangers for xenobiotics because of the counter-current blood flow, thin barrier between internal and external environments, and high rate of both blood and water flow (Satchell, 1984). Xenobiotics are likely taken up from the environment by movement of water across the lamellar “sieve”, followed by diffusion across the gill epithelium from the water into the gill and ultimately ending up in the blood with removal of the chemical once it has reached the blood (McKim, 1991). These processes are directly influenced by the chemistry of the specific organic chemical. McKim and Heath (1983) pioneered an understanding of the relationship between the log K_{ow} of various xenobiotics and their uptake by creating a fish respirometer-metabolism chamber. This chamber provided direct measurements of xenobiotic flux across the gills and indicated that there was a relationship between the log K_{ow} of a chemical and its

absorption rate across the respiratory epithelium in fish (Fig. 1.2). The $\log K_{ow}$, most often referred to as the octanol water partition coefficient, is the ratio of a chemical concentration in the octanol phase (representing lipid solubility) to its concentration in the aqueous phase (representing aqueous solubility). Lipophilic tendencies scale with increasing numbers from -3 to 7 (McKim *et al.*, 1985). Both McKim (1985) and Saarikoski *et al.* (1986) determined that chemicals displaying a $\log K_{ow} < 1$ would tend to load into the fish at a lower rate as these chemicals are partitioned primarily in aqueous media. Chemicals with intermediate $\log K_{ow}$ values (1-3), however, displayed 4-fold greater uptake rates while uptake at higher $\log K_{ow}$ values (3-6) tended to plateau, with uptake rates decreasing at $\log K_{ow}$ values close to 6. This latter effect is likely due to increasing molecular weight and size-dependent diffusion across the gill membrane. Furthermore, the higher the $\log K_{ow}$ value, the more likely the compound will be found bound to a protein carrier. Schmieder and Henry (1988) tested a range of lipophilic xenobiotics with $\log K_{ow}$ values spanning 1-6. They found that increasing $\log K_{ow}$ values of a chemical will increase the likelihood of it being bound to a protein carrier once present in the blood (Fig. 1.3), and thus the percentage bound will increase with increasing K_{ow} values as well. EE2, which is within the range of 3-6, will have a higher percent of binding compared with other less lipophilic xenobiotics. Recently, evidence has appeared that synthetic sex steroids such as EE2 are preferentially taken up across the gills due to their binding affinity for a carrier protein Sex Hormone Binding Globulin (SHBG), located in the filament arteries (Scott *et al.*, 2005). This correlates with the theory that increased K_{ow} will increase the amount of EE2 bound to protein carriers.

Furthermore, uptake of a chemical will be driven by the diffusion gradient with respect to the organism, i.e., moving from an area of high to low concentration until equilibrium is reached (Randall *et al.*, 1998). Ventilation with water and perfusion with blood are likely important in maintaining the diffusion gradient across the gills. In addition, the rate of diffusion will vary with gill plasticity changes (e.g., increases or decreases in diffusion distance or exchange area), ultimately impacting uptake of xenobiotics from the environment (McKim, 1991). All of these processes pertain to oxygen movement across the gills as well, as its uptake also occurs via simple diffusion, and changes in gill plasticity affect this process (Randall *et al.*, 1998). Furthermore, oxygen is considered to be a “lipid soluble” molecule that diffuses across the lamellae transcellularly, which is theorized as the similar pathway for xenobiotics (Yang *et al.*, 2000). These factors bring us back to our previous point, in that any change in the respiratory epithelium, thickness, ventilation and/or perfusion will have a large impact on the exchange of xenoestrogens.

Fundulus heteroclitus as an Ideal Model

Thus, an ideal model organism for bioaccumulation studies is *Fundulus heteroclitus* (killifish), which lives in estuarine environments subjected to a high degree of variability (Burnett *et al.*, 2007). In the wild, killifish are found in tidal marshes and estuaries located along the eastern coast of North America, usually in densely populated areas where exposure to polluted water is not uncommon (Peters *et al.*, 2007). It is a hardy fish

that is key for the trophic dynamics of the ecosystems, where it plays an important role as both predator and prey (Valiela *et al.*, 1977; Kneib *et al.*, 1978; Abraham, 1985; Kneib, 1986; Burnett *et al.*, 2007). In response to these environmental fluxes, killifish have evolved to be highly adaptable and have amazing euryhaline capabilities, as well as thermal and hypoxic tolerance. Another feature making this fish an attractive experimental subject is that its physiology and reproduction have been thoroughly characterized (Burnett *et al.*, 2007). Thus, in recent years *Fundulus heteroclitus* has become increasingly used in the field of toxicology and ecology, due to this organism's extreme adaptability to different environments since they are non-migratory and therefore cope with a wide range of seasonal changes in their natural habitats (Burnett *et al.*, 2007).

Temperature and Gill Epithelia

When fish are faced with environmental changes such as increases or decreases in temperature, they will employ certain strategies to cope with this stress, ultimately altering oxygen uptake at the gills (Nilsson, 2007). Changes in temperature in the aqueous environment directly affects the solubility of oxygen, in that there is a higher carrying capacity for oxygen at lower temperatures, resulting in lower dissolved oxygen in the water at higher temperatures (Sollid and Nilsson, 2006). Furthermore, fish are poikilothermic, indicating that their metabolic rate will directly parallel the temperature of the water. Therefore, since oxygen consumption is a measure of metabolic rate, oxygen consumption will tend to parallel the increases or decreases in the temperature of the surrounding water (Hazel and Prosser, 1974). At higher temperatures, the circulatory

and ventilatory systems must be able to supply sufficient oxygen to sustain increases in biological processes. Oxygen demand is increased at high temperatures to drive aerobic metabolism. Eventually as temperature increases to a critical point (threshold), past the organism's adaptability, then a decline in metabolic rate will ensue at extreme temperatures, decreasing overall performance in fish. The opposite is true for colder temperatures; a decline in temperature will decrease metabolic rate and oxygen consumption. The ability of the mitochondria to produce ATP is decreased in colder temperatures; this compromises the organism's normal physiological function (Portner *et al.*, 2004).

Thus fish, teleosts in particular, have evolved strategies to try to ensure maximal oxygen delivery. Fish can increase oxygen uptake by increasing ventilation rate, by increasing their cardiac output, and/or by increasing their respiratory surface area at high temperatures. The first is accomplished by increasing the rate and stroke volume of buccal pumping, the second by increasing the rate and stroke volume of the heart, and the third is achieved by enhancing lamellar perfusion by increasing arterial blood pressure and hormonal dilation. Resulting in opening a greater proportion of lamellar capillary beds, increasing exchange area, and decreasing mean diffusion distance (Booth, 1978; Davis, 1972; Farrell *et al.*, 1980; Hughes and Saunders, 1970; Taylor and Barrett, 1985). Furthermore, high temperatures affect the gill such that the pillar cells within the lamellae can shrink to decrease diffusion distance for oxygen, and thus the opposite is true under cold temperatures (Sundin and Nilsson, 1998). All of these respiratory strategies are beneficial for an organism to deal with temperature changes; however, because of the

relation of the gill to the environment, any changes in gill morphology to enhance oxygen diffusion across the gills may also allow for elevated toxicant diffusion (Yang *et al.*, 2000). Changes in temperature, and therefore metabolic rate, will likely result in increased EE2 accumulation under high temperatures and decreased accumulation under cold.

Salinity and Gill Epithelia

In addition to temperature, salinity changes can also alter gill epithelia. The gill epithelium of saltwater teleosts is highly permeable to ions. Marine fish are hypo-osmotic relative to seawater; therefore, they will lose water by osmosis and gain Na^+ and Cl^- by osmosis. Gill epithelia contain MRCs of the type generally referred to as saltwater chloride cells (SWCC) or just chloride cells. These cells control osmoregulation through active ion secretion, excreting Na^+ and Cl^- across the gills to the external seawater (Kaneko and Katoh, 2004; Marshall, 1995). In saline environments, killifish must also drink salt water in order to replace water lost across the gills by osmosis, thus these secretory cells are important for removal of both the excess salt gained through drinking and the salt gained by passive uptake from the saline environment (Ramachandran *et al.*, 2006). In contrast, fish in fresh water are hyper-osmotic, so they must actively take up salt from the environment, as well as excrete, via the kidney, the excess water that enters the body via osmosis. They also restrict drinking, because it would lead to further accumulation of excess water.

There are also specialized gill transport cells to counteract ion loss in most freshwater fish. These MRCs, generally referred to as freshwater chloride cells (FWCCs), pump ions from the dilute external environment into the fish. It remains controversial whether *Fundulus heteroclitus* actually possesses FWCCs (Katoh *et al.*, 2001, Katoh and Kaneko, 2004; Laurent *et al.*, 2006), because the killifish does not take up Cl^- at the gills in fresh water (Patrick *et al.*, 1997; Wood and Laurent, 2003) and the morphology of the putative MRCs appears unusual. Laurent *et al.* (2006) have termed these “cuboidal cells” and have proposed that they actively take up only Na^+ , and not Cl^- , from fresh water. Regardless, the structure of the gill is very different between seawater- and freshwater-adapted killifish.

Thus, the gills of any teleosts are the principal sites for ionoregulation, and changes in ion fluxes will take place depending on the salinity of the surrounding aquatic environment (Ramachandran *et al.*, 2006). Increases or decreases in salinity will alter the overall gill structure such that the amount of ion-pumping “chloride cells” (noted for their thickness) will alter the diffusion distance for oxygen, depending on the salinity (Nilsson *et al.*, 2007). The cost of ionoregulation is likely to vary with the intensity of the water-to-blood osmotic and electrochemical gradients for ions. As the osmolarity of fish blood is similar to ~ 30% seawater, these gradients and costs will be greatest in 100% sea water, least at the isosmotic point (30% seawater), and intermediate in fresh water (Nagibina, 1983; Morgan and Iwama, 1991). Furthermore, this rearrangement of cells within the gill to deal with different salinities also has an energy cost. Several studies have indicated that the destruction and creation of new cells in different salinities is a metabolic drain

(Yung Che and Hwang, 2008). Since osmoregulation directly affects energetic demands, oxygen uptake will increase to meet this need. Salinity will furthermore cause structural rearrangements that could potentially alter the diffusion distance for oxygen. In addition, under osmoregulatory stress fish employ strategies for increasing oxygen contact at the gill that are similar to the strategies utilized when exposed to high temperatures, such as increasing perfusion and ventilatory processes. These processes are adaptive for energy compensation, but also increase the probability of toxicant uptake.

In summary, any environmental stressor which 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.

Objectives

The aim of my study was to investigate the rate of uptake of 17 α -ethynylestradiol (EE2) – a potent endocrine disrupting substance in – *Fundulus heteroclitus*, under conditions of varying temperatures and salinities. The ultimate goal, is to produce a model for uptake of EE2 under changing environmental conditions reflecting that of an estuarine, tidal environment where both temperature and salinity fluctuate diurnally and seasonally. The practical consequence is to supply guidance for the siting and timing of sewage treatment plant discharges in estuaries, relative to salinity and thermal regimes, to minimize EE2 uptake in wild populations. To fulfill this goal, my specific objectives were:

- 1) To expose killifish to radio-labeled EE2 at a nominal waterborne concentration of 100ng/L under varying conditions of both temperature and salinity, and to assess the uptake rate under each of these conditions;
- 2) To investigate the possible relationship between oxygen consumption rate and EE2 uptake rate under these environmental conditions;
- 3) To capitalize on the radio-labeled compound to assess the tissue-specific distribution of EE2 under different environmental conditions;
- 4) To test other species of teleosts – specifically rainbow trout – to see if uptake of EE2 is similar across species.

In this investigation, I tested the following hypotheses:

Hypothesis 1) Increases in temperature will result in increases in the uptake rates of both oxygen and EE2 in killifish.

Hypothesis 2) Salinity will significantly alter the uptake rate of EE2 in killifish.

Hypothesis 3) Oxygen uptake rate (consumption) will be a strong predictor of EE2 uptake rate in killifish under conditions of both temperature and salinity manipulations.

Figure 1.1: The structure of two estrogenic steroids: E2(17- β -estradiol), naturally occurring– EE2(17- α -ethynylestradiol) a synthetic. Reproduced from Huber *et al.* (2004).

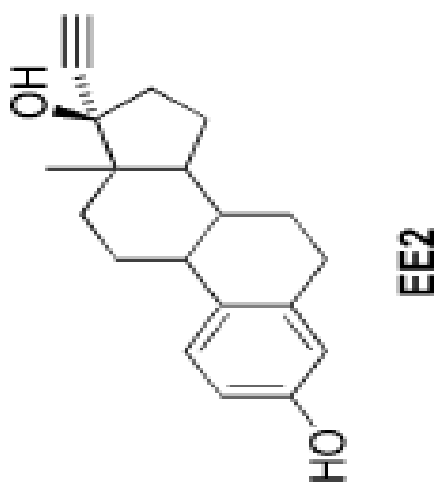
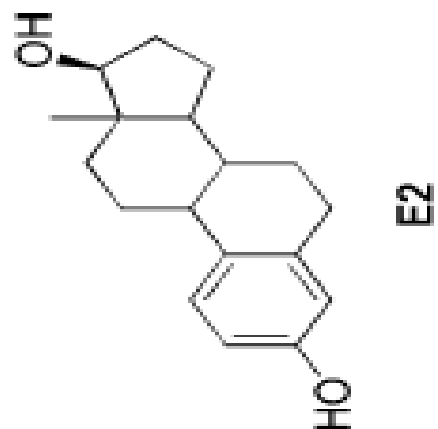


Figure 1.2 Relationship between gill absorption and log K_{ow} in rainbow trout and guppies in an exposure to various hydrophobic organics at sub-lethal exposures. Rainbow trout sub-lethal exposures 1 = benzaldehyde; 2 = 2,4-dinitrophenol; 3 = MS-222; 4 = malathion; 5 = 1-octanol. b, guppy sub-lethal exposures: 1 = butyric acid; 2 = phenol; 3 = benzoic acid; 4 = 4-phenylbutyric acid; 5 = 2, 4-dichloro-phenol; 6 = 2-sec butyl-4, 6-dinitrophenol; 7 = 3, 4-dichlorobenzoic acid; 8 = 2, 6-dibromo-4-nitrophenol; 9 = 2, 4,5-trichlorophenol; 10 = 2,4,6-trichlorophenol; 11 = 2,3, 4, 6-tetrachlorophenol; 12 = tetrachloroverathrol; 13 = pentachlorophenol; 14 = pentachloroanisole; 15 = 2,4,6-trichloro-5-phenylphenol; 16 = DDT; and 17 = 2,3, 6-trichloro-4-nitrophenol.

Reproduced from McKim *et al.* (1985) Saarikoski *et al.* (1986)

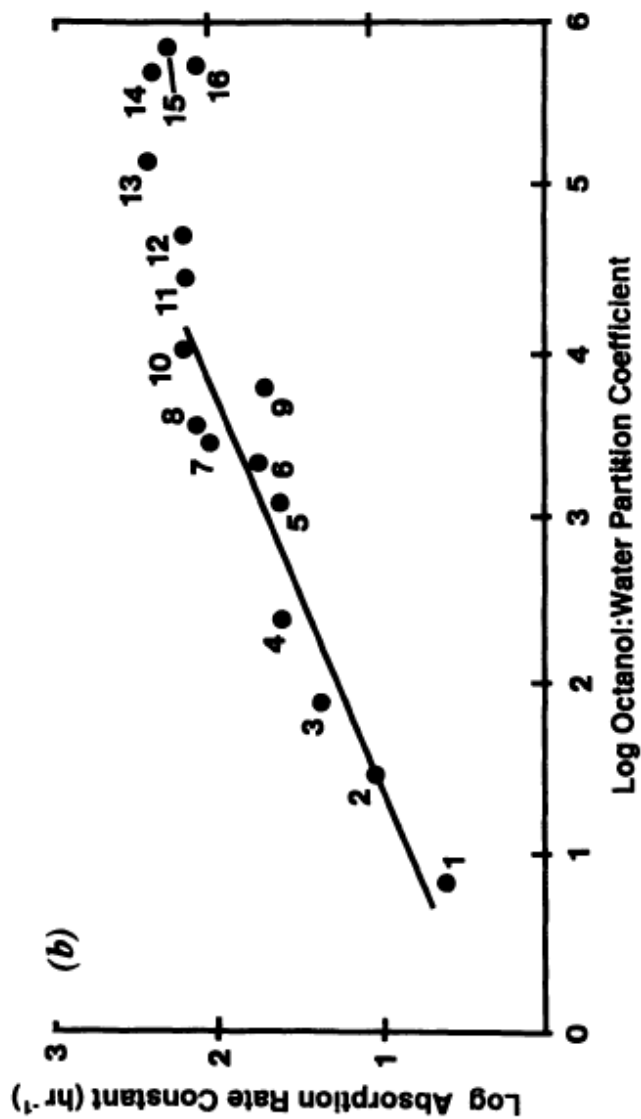
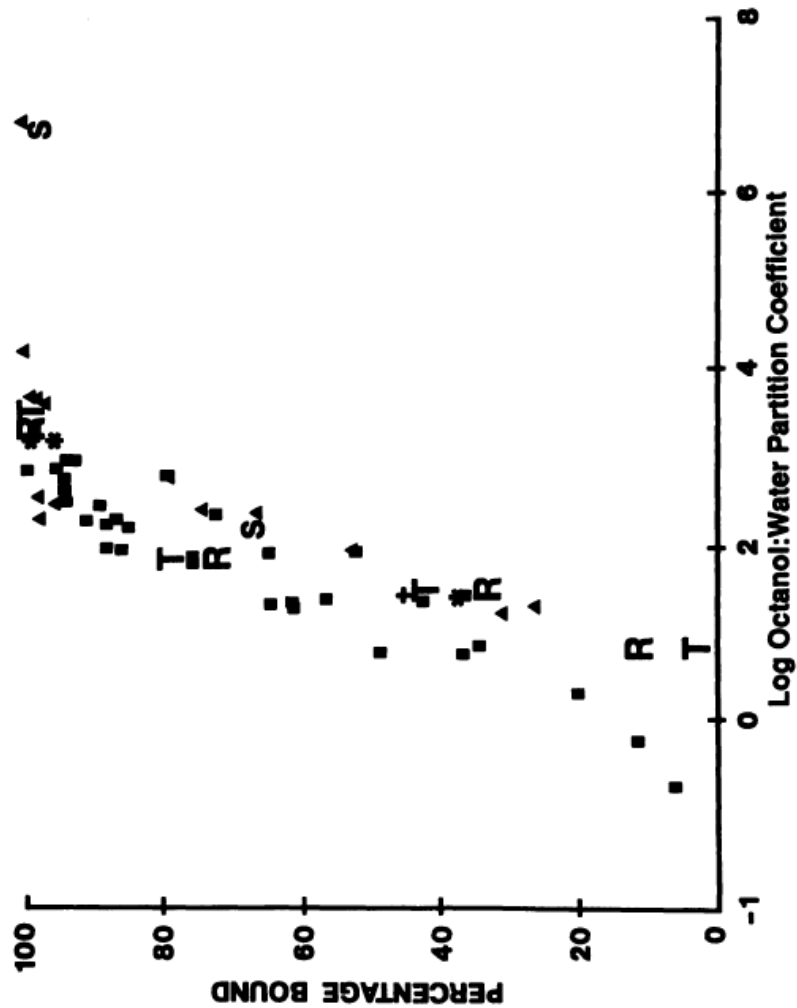


Figure 1.3 Mean percentage of hydrophobically-bound organics in rainbow trout(T), shark(S) and rat(R) to blood plasma proteins such as bovine serum, albumin, and human serum albumin. Reproduced from Schmieder and Henry (1988).



CHAPTER 2: ENVIRONMENTAL INFLUENCES ON 17- α ETHYNYLESTRADIOL UPTAKE AND THE RELATIONSHIP TO OXYGEN CONSUMPTION IN THE MODEL EURYHALINE TELEOST (*Fundulus heteroclitus*)

Abstract

The synthetic estrogen 17 α -ethynylestradiol (EE2), an endocrine disruptor originating from birth control and hormone replacement therapy, is discharged in wastewater treatment plant (WWTP) effluents. The present study employed radio-labeled EE2 to examine the impact of temperature and salinity on the uptake of EE2 in male killifish (*Fundulus heteroclitus*), a model euryhaline teleost. Fish were exposed to a nominal concentration of 100 ng/L EE2 for 2 h. Actual concentrations were lower due to EE2 adsorption to the exposure system, but uptake rates were normalized to 100 ng/L. Oxygen consumption rates (MO₂), whole body EE2 uptake rates, and tissue-specific EE2 distribution were monitored. EE2 uptake by freshly killed fish was negligible. In killifish acclimated to 18°C at 16 ppt (50 % seawater), MO₂ and EE2 uptake were both much lower after 24-h exposure to 10°C and 4°C, and increased after 24h exposure to 26°C. Transfer of killifish to fresh water for 24 h tended to lower EE2 uptake rate, and long-term acclimation to fresh water reduced it by about 70 %. Long-term acclimation to 100 % sea water (32 ppt) also reduced EE2 uptake rate by about 50 % relative to 16 ppt. However, this was not seen in juvenile rainbow trout (*Oncorhynchus mykiss*) where uptake rates were the same in FW- and 16 ppt-acclimated trout. The tissue-specific accumulation of EE2 was found to be the

highest (40-60% of the total) in the liver plus gall bladder across all exposures, and the great majority of this was in the bile in killifish, regardless of temperature or salinity, whereas in trout accumulation was the highest in the carcass at 70 % of the total. The carcass was the next highest accumulator (30-40 %) in killifish, followed by the gut (10-20 %) with only small amounts in gills and spleen. Drinking rate, measured with radio-labeled polyethylene glycol-4000, was about 25-times greater in 16 ppt-acclimated killifish relative to freshwater-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 pathways for uptake across the gills of these lipophilic molecules. These data will be useful in developing a predictive model of how variations in key environmental parameters (salinity, temperature, dissolved oxygen) affect EE2 uptake in estuarine fish, so as to determine optimal timing and location of WWTP discharges.

Introduction

Exposure to elevated levels of natural and synthetic estrogens can cause altered endocrine function and decreased reproductive success in fish (Dube and MacLachy, 2001). Many of these natural and xenoestrogens are discharged into receiving fresh water and estuarine systems through wastewater treatment plants (WWTP) and by industrial effluents (Langston *et al.*, 2005). These xenoestrogens are known to mimic and antagonize endogenous hormones, thereby disrupting their synthesis, degradation and metabolism (Langston *et al.*, 2005; Thorpe *et al.*, 2003). Examples include the 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 estradiol (E2). EE2 is most commonly used in the female oral contraceptive pill and in hormone replacement therapy in menopausal women (Ternes *et al.*, 1999; Thorpe *et al.*, 2003; Peters *et al.*, 2007). 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 bioconcentrate in aquatic ecosystems due to its longer half life (Tyler *et al.*, 1998; Langston *et al.*, 2005). Indeed, in laboratory studies EE2 can cause estrogenic effects when present in the range of 1-10 ng/L. However, higher levels have been documented in Canadian sewage outflows (Ternes *et al.*, 2007) and once these chemicals are 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 ecosystems (Valiela *et al.*, 1977; Kneib *et al.*, 1978; Abraham, 1985; Kneib, 1986). Endocrine disrupting effects of EE2 exposure on this species are already well-documented (Dubé and MacLatchy, 2001; MacLatchy *et al.*, 2003; MacLatchy *et al.*, 2005; Peters *et al.*, 2007, Peters *et al.*, 2010). Killifish move diurnally to and from tidal margin shallows, where salinity, temperature and dissolved oxygen are constantly varying (Dubé and MacLatchy, 2001). Increased understanding of its ionoregulatory, reproductive and developmental physiology, together with rapid progress on its genome, has made *Fundulus heteroclitus* a model estuarine teleost (Burnett *et al.*, 2007).

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 under standardized conditions in strictly freshwater environments. The actual 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. 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 will require changes in gill ventilation, perfusion and functional surface area. Thus, we hypothesized those environmental influences that affect oxygen transfer across the gills (oxygen consumption) will also affect the uptake and bioaccumulation of EE2. Temperature is one such variable, so we assessed the impact of four representative temperatures (4, 10, 18, and 26°C) on EE2 uptake, using radio-labeled 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 their 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 radio-labeled 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 radio-labeled compound was that it allowed us to assess the short-term tissue-specific disposition of EE2 after uptake. Finally, we also examined EE2 uptake in the rainbow trout (*Oncorhynchus mykiss*), to evaluate whether the same principles applied in another euryhaline teleost.

The present study is unique in that we have measured acute uptake rates of an endocrine disrupting substance under different environmental influences accurately representing natural environmental conditions. The eventual goal of this research program is to develop a predictive model of how variations in key environmental parameters (salinity, temperature, dissolved oxygen) affect EE2 uptake in estuarine fish, so as to provide guidance for the location and timing of WWTP discharges.

Materials and Methods

Fish Husbandry

Killifish

Adult killifish *Fundulus heteroclitus* (1-5 g) were obtained by seining from an uncontaminated site: Horton's Creek in Miramichi, New Brunswick, Canada, in June of 2009 and the Bay of Fundy in August of 2010. Fish were then transferred to McMaster University (Hamilton, Ontario, 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, or 100 % sea water (32 ppt) at 18°C for at least 3 weeks prior to experimentation. Fresh water was dechlorinated Hamilton, Ontario tap water (moderately hard: $[\text{Na}^+] = 0.6$ mequiv/L, $[\text{Cl}^-] = 0.8$ mequiv/L, $[\text{Ca}^{2+}] = 1.8$ mequiv/L, $[\text{Mg}^{2+}] = 0.3$ mequiv/L, $[\text{K}^+] = 0.05$ mequiv/L; titration alkalinity 2.1 mequiv/L; pH ~8.0; hardness ~140 mg/L as CaCO_3 equivalents). Saline waters were made by the addition of Instant Ocean salt (Big Al's Aquarium Supercenter, Woodbridge, ON, Canada) to fresh water. All aquaria were set up with re-circulating pumps which 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 12h:12h light: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.

Rainbow Trout

Juvenile rainbow trout *Oncorhynchus mykiss* (1-3 g) were obtained from Humber Springs trout farm (Orangeville, Ontario, Canada) and acclimated to the same two salinities, but at 13°C, their preferred temperature. Freshwater trout were held in 500-L flow-through tanks containing Hamilton dechlorinated tap water. For 50 % seawater acclimation, trout were held in 40-L tanks with a charcoal filter with water changes every 2-3 days, and over a period of 4 weeks, were acclimated to a salinity of 16 ppt by

increasing the salinity by 10 % every other day, until 16 ppt was obtained, and then fish were then held at this salinity for 18 days before experimentation. Both acclimation groups were fed every day with a 1 % body mass ration of Martin's dried commercial trout pellet feed(1 point) (Martin Mills Inc, Elmira, ON, Canada) and were subjected to a 12 h:12 h light:dark daily photoperiod.

Oxygen Consumption and EE2 Uptake Experiments

During exposures killifish were held in individual custom-made, shielded respirometers, filled initially with either (i) reference condition water (16 ppt at 18°C) for most experiments or (ii) fresh water (0 ppt) at 18°C for FW-acclimated fish, and iii) for salinity transfer experiments only, killifish were taken from the 16 ppt acclimation tank and placed in FW for 24 h prior to experiments. 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 so that metabolic rate was normalized across all animals.

After 24 h, the water was gently replaced, and the aeration stone was removed. The water was then dosed with radio-labeled [³H] - ethynylestradiol, obtained from

American Radiolabeled Chemicals (St. Louis, MO, USA) and used at a specific radioactivity of 2.024×10^{-7} Ci/ μ g 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-radio-labeled EE2 (Sigma Aldrich, 98% HPLC grade) to the radio-labeled 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 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 cold EE2 (Sigma Aldrich, 98% HPLC grade) at a concentration of 10 μ g/L for 5 min, to displace any radio-labeled EE2 that was loosely adsorbed by the body surface. After 5 min, fish were euthanized with a lethal dose of NaOH-neutralized MS-222 (Syndel Laboratories Ltd., Vancouver, B.C., 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.

Dead Fish Control Experiment

Fish acclimated to 16 ppt at 18°C were terminally euthanized with a lethal dose of NaOH-neutralized MS-222 for 30 min prior to experimentation. Euthanized fish were then placed in individual respirometers (16 ppt, 18°C), and dosed in a similar manner to

above. An aeration stone provided thorough mixing throughout the experiment. The exposure procedure followed exactly as above, with water samples taken at 0, 60 and 120 min, and the fish were similarly harvested at the end of the 2-h experimental exposure.

Rainbow Trout EE2 Uptake Experiments

Rainbow trout exposures followed the same protocol as the killifish experiments. Rainbow trout were held in the same individual custom-made, shielded respirometers (516 ml), filled initially with either 50 % sea water (16 ppt at 13^oC) or fresh water (at 13^oC). Once fish were placed in the respirometers, the units were moved to a constant temperature water bath (13^oC). The water was vigorously aerated throughout this adjustment period. Trout were also fasted during this 24-h period so that metabolic rate was normalized across all animals. After the 24 h, the exact protocol for killifish was followed.

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^oC for 8 h in the previously-mentioned water bath system. After a 2-h settling period, a dose of 8 μ Ci radiolabelled [³H]-polyethylene glycol, M.W. 4000 (PEG-4000; Perkin Elmer Life and Analytical Sciences, Boston MA, USA) with a specific activity of 1.28mCi/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 EthiconTM 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.

Tissue Analyses

The same basic methods were used for both radio-labeled [³H]-EE2 and radio-labeled [³H]-PEG-4000. Each organ was weighed; the carcass was placed in a 50-ml CorningTM 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 1N trace metal grade nitric acid (Sigma-Aldrich, St. Louis, MO, USA) at volumes of 3-5 times (exact volume recorded) the weight of the organ, except for the gut which was digested in 2N trace metal grade nitric acid. The sealed vials were placed in an incubator at 65^oC for 48 h, with vigorous vortexing at 24 h. The digested samples were then centrifuged for 5 min at 3500 rpm at 18^oC. 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 fluor (Ultima Gold, Perkin Elmer, Waltham, MA, USA). The tissue samples were counted on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer, Waltham, MA, USA), 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.

Water Analyses

Water PO₂ was measured using a Clarke-type oxygen electrode (Cameron Instruments) connected to an AM Systems Polarographic Amplifier (model 1900, Carlsborg WA, USA) digital dissolved oxygen meter. The electrode was maintained and calibrated at the chosen experimental temperature. Water radioactivities of either [³H]-EE2 or [³H]-PEG-4000 were measured by adding 1-ml water samples to 3 ml of scintillation fluor (Opti-phase, Perkin, Elmer, Waltham, MA, U.S.A.), and counting on the same liquid scintillation analyzer as used for tissue samples, with all values quench-corrected to a common counting efficiency.

Calculations

Oxygen consumption (MO₂) was calculated using the equation below where change in water PO₂ per unit time was multiplied by the O₂ solubility coefficient (Boutilier *et al.*, 1984), then factored by respirometer volume and normalized to a 5-g fish.

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

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 ($\mu\text{mol/L/mmHg}$), 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 5g fish, and expressed as $\mu\text{mol/5-g fish/h}$, so as to remove the 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 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. 2.1, Results). To correct for this, all rates were adjusted to an EE2 exposure concentration of 100 ng/L.

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

Where $\text{CPM}_{\text{total}}$ is total counts per minute of EE2 in the whole body of the fish, W_f 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 W 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:

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

Q10 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 and EE2 uptake at temperatures of t_1 and t_2 respectively (Schmidt-Nielsen, 1997).

Drinking rates (DR, in ml/kg/h) were 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:

$$DR = \frac{CPM_{(totalgut)}}{CPM_{(water)}} \times \frac{1}{\Delta t} \times \frac{1}{W_f} \quad (5)$$

Where $CPM_{(total\ gut)}$ is the total amount of counts per min present in the gut, Δt is the flux time; $CPM_{(water)}$ is the average counts per min per ml of water and W_f 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:

$$\text{Theoretical uptake rate by drinking} = DR \times W_{cpm} \times 1/SA \quad (6)$$

Where DR is the actual drinking rate that was measured (calculated for a 5-g fish in 16 ppt), W_{cpm} 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.

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, an 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 Fisher LSD *post hoc* test or, in the case of a failed normality test, an ANOVA on ranks analysis, followed by a Dunn's *post hoc* test. 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 ($P < 0.05$) from one another. Figure legends denote the specific test performed for each trial.

Results

EE2 Exposure Levels

Mean exposure levels during the various trials are shown in Fig. 2.1. In different series, mean concentrations ranged from about 43 to 65 ng/L, and tended to decline slightly over time. As the initial nominal addition was sufficient to create a concentration of 100 ng/L, there was clearly an immediate loss to the walls of the respirometers, followed by a small subsequent loss likely attributable to uptake by the fish (see Discussion). Therefore, all EE2 uptake rates were adjusted to an exposure concentration of 100 ng/L as outlined in Methods.

EE2 Uptake Rates of Dead Fish

Experiments with recently deceased killifish under reference conditions (18°C, 16 ppt) revealed negligible EE2 uptake rates, close to the limits of detection, 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 2.1), less than 4 % of the rate measured in live killifish under the same conditions (Fig. 2.2A).

EE2 and MO₂ 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. 2.2A). Uptake rates, however, increased to 1.20 ng/5-g fish/h when temperature was increased to 26°C (Fig. 2.2A). A similar pattern was seen in MO₂, 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. 2.2B). Q10 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 Q10 values for MO₂ were high (4.42) only in the 10-18°C range. For both EE2 uptake and MO₂, Q10 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 MO₂ ($r^2=0.95$, $p<0.05$; Fig. 2.2C).

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, 16ppt) accumulated EE2 at a 3-fold greater rate than freshwater-acclimated killifish, 2-fold greater than 100% seawater-acclimated animals (32 ppt), and nearly 1.5-fold greater rate than killifish transferred to fresh water for 24 h prior to exposure, all at the same temperature (Fig.

2.3A). In contrast, there was no significant difference in whole body EE2 uptake rates between freshwater-acclimated rainbow trout and 50% seawater-acclimated rainbow trout, both tested at 13°C. (Fig. 2.3B). Unlike the temperature series (Fig. 2.2C), there was not a significant positive correlation ($r^2=0.40$, $p=0.175$) between EE2 uptake and MO_2 in this series (Fig. 2.3C). Even when the trout data were excluded, the relationship remained non-significant ($r^2=0.60$, $p=0.122$).

Tissue-specific Accumulation of EE2 in Killifish and Rainbow Trout

Following a 2-h exposure to 100 ng/L of radio-labeled 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. 2.4A,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, 16ppt), the gall bladder usually accounted for about 35% of the total, while the liver accounted for 25 % of the radio-labeled EE2 (Fig. 2.4B). 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.

However, a dissimilar tissue-specific pattern was seen in the trout salinity series at 13°C (Fig. 2.4C). Unlike killifish, the dominate site of accumulation in trout was the

carcass, accounting for about 70 % of the total. The liver accounted for < 20 % and when the liver and gall bladder were isolated, these organs accounted for approximately 10 % each, as illustrated for the freshwater-acclimated trout at 13°C. All other organs accounted for < 10 % of the total disposition (Fig. 2.4D).

Drinking

As the gut tissues accounted for 10-20 % of the total EE2 accumulation after 2 h in the killifish (Fig. 2.5 A, 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 16ppt, approximately 25 times higher than the very low rates (~ 0.4 mL/kg/h) in freshwater-acclimated animals (Fig. 2.5A). When the drinking rate at 16 ppt was used to predict the EE2 bioaccumulation seen in the gut (see equation 6, calculation section of Methods), it accounted for about 30% of the actual measured value (Fig. 2.5B), or less than 5 % of the whole body uptake.

Table 2.1: EE2 uptake rate (ng/5-g fish/h) in recently deceased killifish (*Fundulus heteroclitus*) at 18°C and 16 ppt. Means $\pm$ S.E.M (N = 5).

Organ	EE2 Uptake Rate (ng/5-g fish/ h) in Dead Fish
Carcass	0.046 $\pm$ 0.01
Gut	0.005 $\pm$ 0.001
Gill	0.002 $\pm$ 0.0006
Liver + Gall bladder	0.004 $\pm$ 0.02
Spleen	0.0005 $\pm$ 0.0001
Total	0.058 $\pm$ 0.01

Figure 2.1: 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 radio-labeled 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 $\pm$ S.E.M (N= 4-6 per treatment and 10 for 32 ppt).

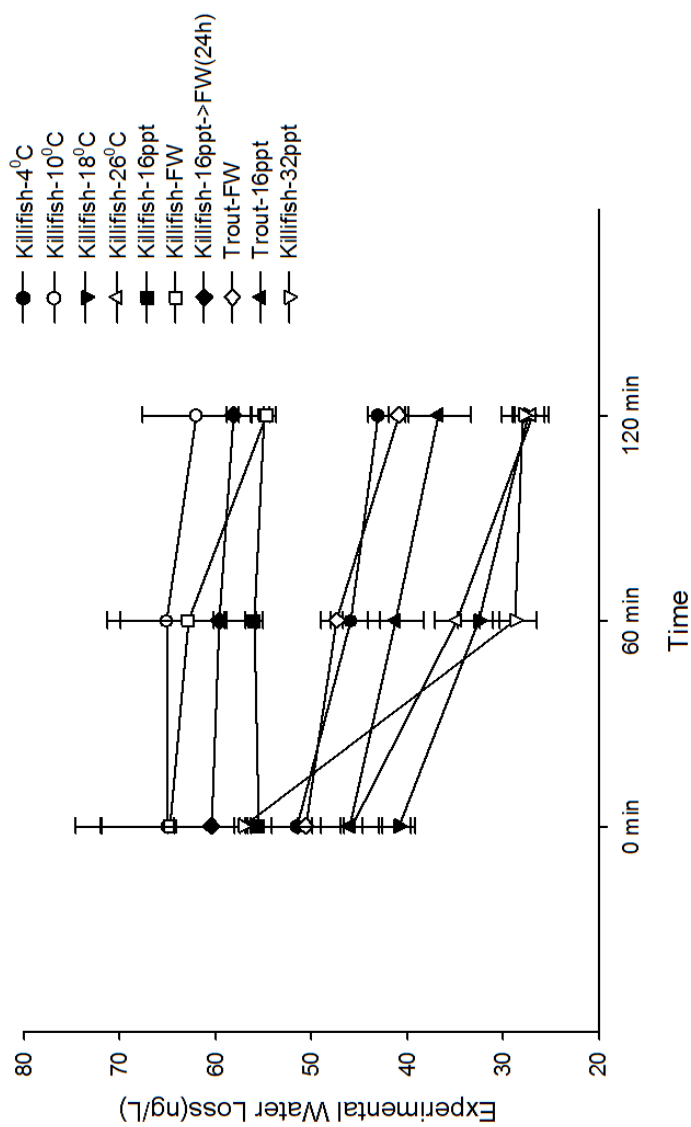


Figure 2.2 (A). Whole body EE2 uptake (ng/5-g fish/h) in killifish (*Fundulus heteroclitus*) acclimated to 18°C at a salinity of 16 ppt, then exposed for 24-h to temperatures of 4, 10, 18, and 26 °C at the same salinity. The Q10 values for the various temperature intervals are shown. Values are means $\pm$ S.E.M. (N= 5 per treatment, except the 18°C treatment with an N=6 and 4°C with an N = 4). Means with different letters are significantly different ($P < 0.05$) as determined by an ANOVA on ranks followed by Dunn's *post hoc* test.

(B). Simultaneous rates of O₂ consumption (MO₂, μ mol/5-g fish/h) measured in the same treatments as panel A. The Q10 values for the various temperature intervals are shown. Means with different letters are significantly different ($P < 0.05$) as determined by an ANOVA followed by Fisher's LSD *post hoc* test.

(C). The overall relationship between whole body EE2 uptake and MO₂ ($r^2=0.95$, $p < 0.05$) at different temperatures in the treatments of panels A and B.

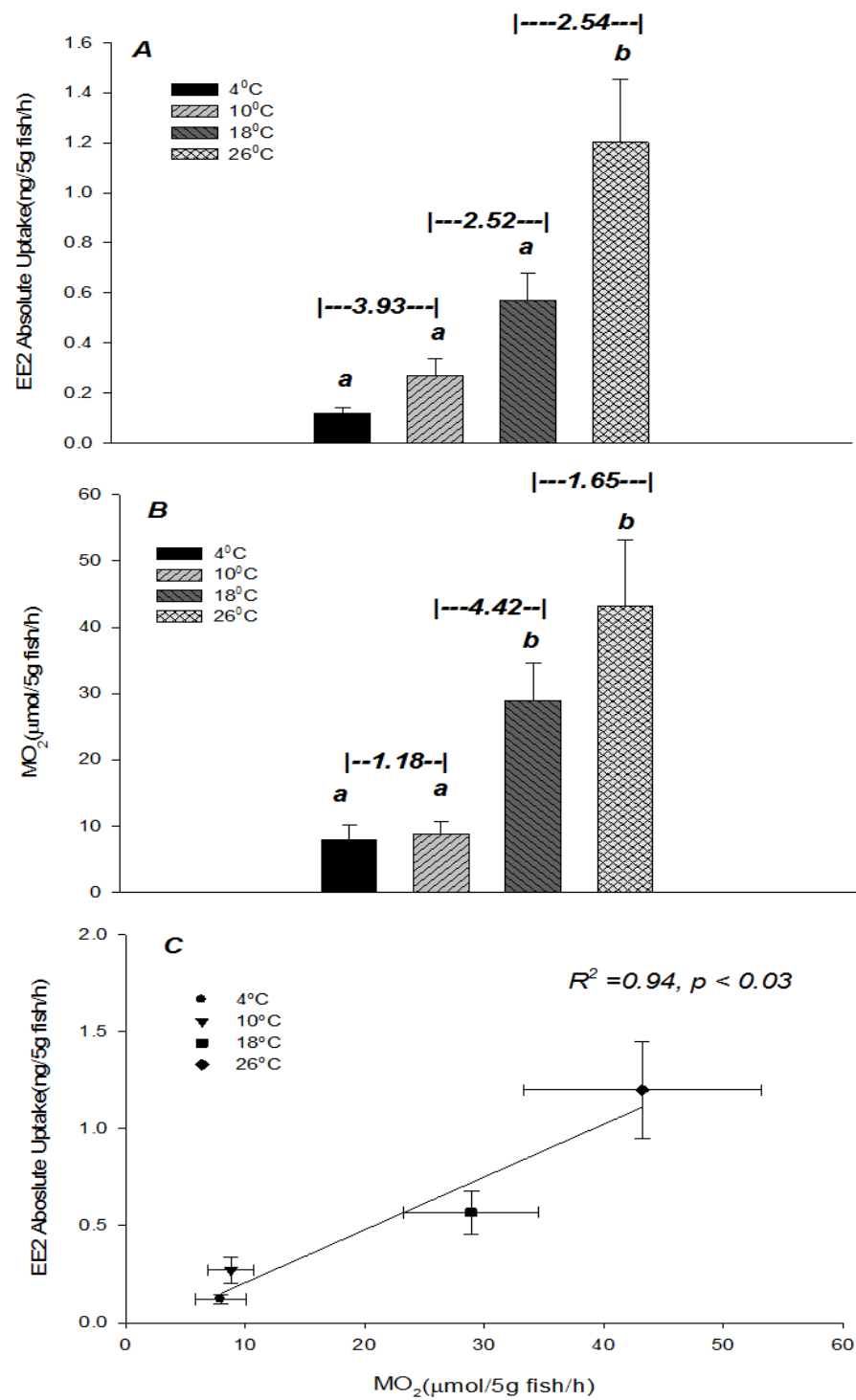


Figure 2.3. (A). Whole body EE2 uptake (ng/5-g fish/h) of killifish (*Fundulus heteroclitus*) exposed to four different salinity conditions: freshwater acclimation (0 ppt), 16 ppt acclimation (50 % sea water), 32ppt acclimation (100 % sea water), or 24-h transfer to fresh water after acclimation to 16 ppt, all at 18°C. Values are means $\pm$ S.E.M. (N= 5 per treatment, except 32 ppt with an N=10). Values not sharing the same letter are significantly different ($P < 0.05$) as determined by an ANOVA on ranks followed by a Dunn's *post hoc* test.

(B). Whole body EE2 uptake (ng/5-g fish/h) in rainbow trout (*Oncorhynchus mykiss*) exposed to two different salinity conditions: freshwater acclimation (0 ppt) or 16 ppt (50 % sea water) at 13°C. Values are means $\pm$ S.E.M. (N= 6 per treatment), and were not significantly different (Student's t-test, $P > 0.05$).

(C). The overall relationship between the whole body EE2 uptake and the simultaneous rates of O₂ consumption (MO₂) measured in the various salinity treatments of panels A and B ($r^2=0.40$, $p=0.175$) at different temperatures in the treatments of panels A and B. Values are means $\pm$ S.E.M. (N= 5 per treatment for killifish, unless otherwise stated and N=6 per treatment for rainbow trout unless otherwise stated).

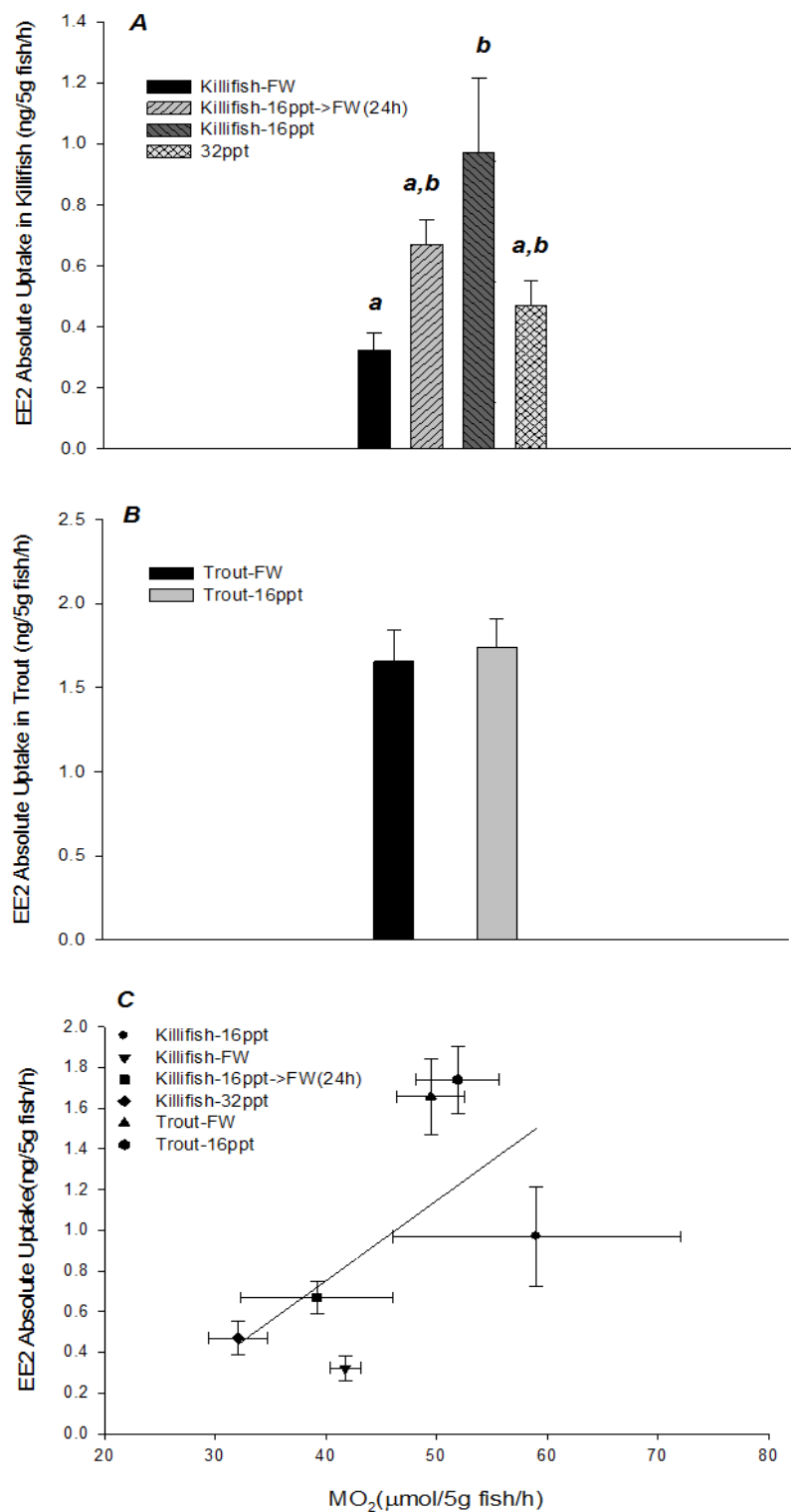


Figure 2.4. (A). EE2 uptake in specific organs, normalized to a 5 g fish (ng/5-g fish/h), in killifish (*Fundulus heteroclitus*) exposed to temperatures ranging of 4, 10, 18, and 26 °C at a salinity of 16ppt. Values are means $\pm$ S.E.M. (N= 5 per treatment, except the 18 °C and 4 °C treatments where N=4). Note that the ‘liver’ contains both the liver and the gallbladder.

(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 $\pm$ 1 S.E.M. (N = 4 per treatment).

(C). EE2 uptake in specific organs, normalized to a 5 g fish (ng/5-g fish/h), in rainbow trout (*Oncorhynchus mykiss*) exposed to two different acclimation conditions, fresh water (0 ppt) and 16 ppt (50% sea water) at 13 °C. Means $\pm$ 1 S.E.M. (N = 6 per treatment of freshwater and N=5 for fresh water). Note the ‘liver’ contains both the liver and gallbladder.

(D). EE2 uptake expressed as a percentage of total whole body uptake rates in the organs of rainbow trout (*Fundulus heteroclitus*) exposed to 13 °C in fresh water. Means $\pm$ 1 S.E.M. (N = 6 per treatment). Note that the gall bladder and liver are measured separately.

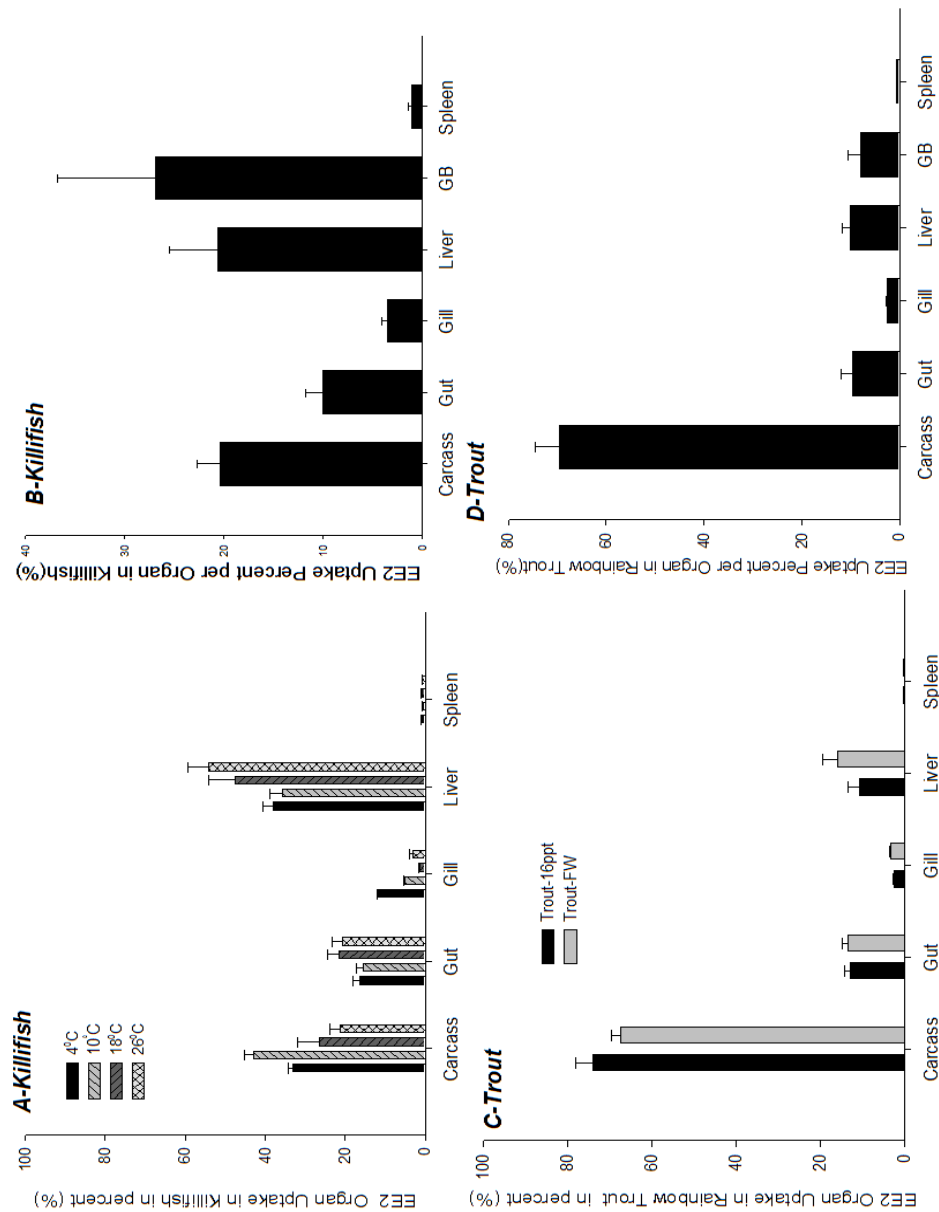


Figure 2.5(A). Drinking rates (mL/kg/h) measured in killifish (*Fundulus heteroclitus*) acclimated to a salinity of 16 ppt or freshwater. Values are means $\pm$ S.E.M (N= 5 per treatment). Asterisk indicates significant difference ($P < 0.05$) by Student's t-test.

(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 Methods for details on calculations.

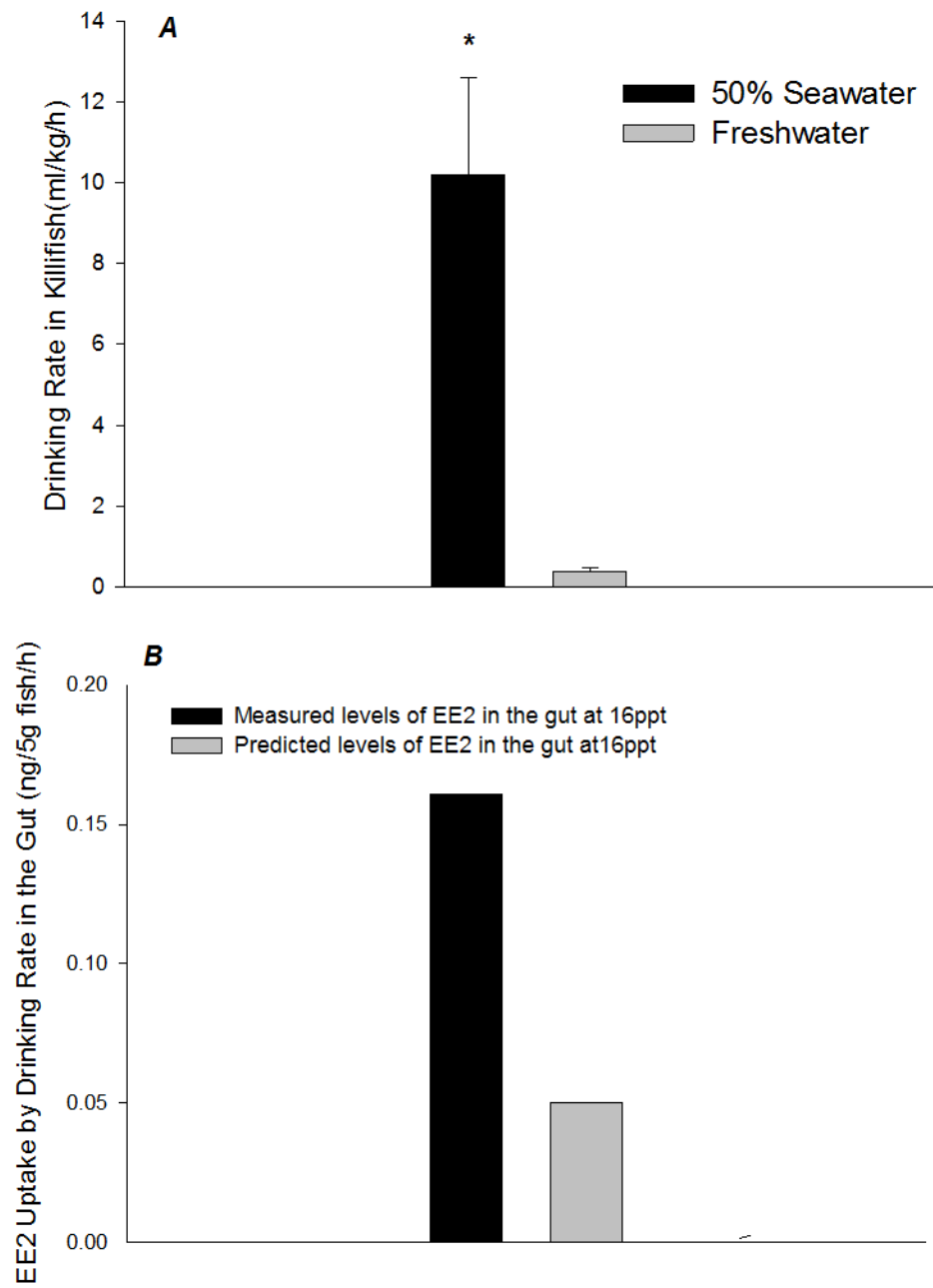
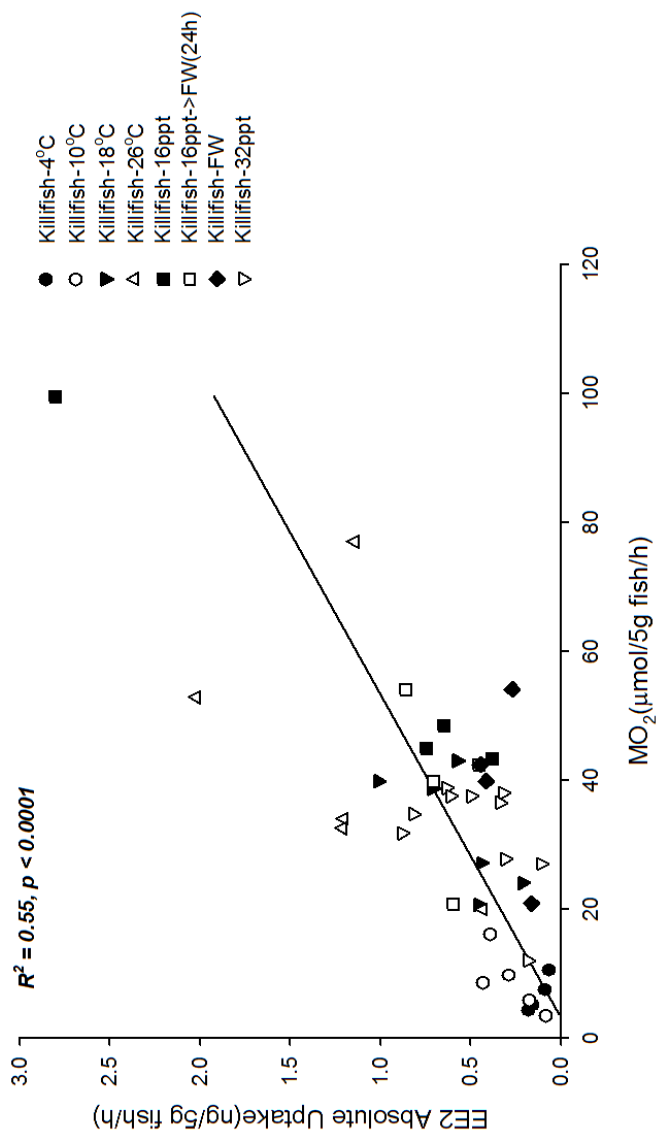


Figure 2.6: Overall relationship between the rate of oxygen consumption (MO_2) and the rate of whole body EE2 uptake in individual killifish and rainbow trout, with temperatures ranging from 26 °C to 4 °C, and salinities of freshwater, 16 ppt, and 32 ppt in the various experimental series. $R^2 = 0.55$, $p < 0.0001$



Discussion

As hypothesized, we demonstrated that changes in uptake of EE2 occurred under differing environmental parameters. 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 changes and physiological responses to the influences that affect both oxygen consumption and the bioavailability of this xenoestrogen.

EE2 Concentrations

The waterborne concentration of EE2 was initially lower than the added dosing of 100 ng/L (Fig. 2.1). Most of the loss was immediate (i.e prior to 0 min), indicating adsorption to 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.

Temperature and EE2

At higher environmental temperatures, the carrying capacity of water for oxygen is decreased, resulting in less dissolved O₂ for any given PO₂. 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 rate of the heart (i.e., perfusion) (Randall, 1982). Fish will also enhance lamellar perfusion by increasing arterial blood pressure and hormonal dilation, thereby opening a greater proportion of lamellar capillary beds (Hughes and Saunders, 1970; Davis, 1972; Booth, 1978; Farrell *et al.*, 1980; Taylor and Barrett, 1985). The respiratory surface area, therefore, becomes enlarged and the diffusion distance thinned (Yang *et al.*, 2000; Nilsson *et al.*, 2007). While these strategies will enhance oxygen uptake, they may also increase EE2 uptake. Thus, it is not surprising that that highest uptake of EE2 occurred at the highest temperature (26°C, 16 ppt) (Fig. 2.2C). 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. 2.2C).

The Q10 value is a commonly-used index that quantifies the rate at which processes change with temperature. If the Q10 values are below 1.5, passive processes are predominating, i.e., simple diffusion. However, if the Q10 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 Q10 values were above 2.0 indicating that uptake is very dependent on temperature, displaying rates of reactions increasing 2-3 fold as temperature increases 10-fold.

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 2.1). Thus, EE2 accumulation in live fish was not by surface adsorption, but rather by a specific uptake pathway, resulting in accumulation in the internal organs of live fish.

The gills are the likely entry point for EE2 uptake, because of their lipid-rich composition and thin barrier between the aqueous environment and the blood. The diffusion of EE2 across the branchial epithelium is thought to occur transcellularly through a form of passive diffusion like oxygen (Murphy and Murphy, 1971; Hunn and Allen, 1974; Satchell, 1984; McKim, 1991; Brauner *et al.*, 1994; Yang and Randall, 1995; Yang *et al.*, 2000), so EE2 likely diffuses through the gill as opposed to accumulating in this tissue. This may implicate entrance at the gills as the rate limiting step. Our results support this notion as only low levels of EE2 were repeatedly observed in the killifish gill after acute exposures (Fig. 2.4 A, B). 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; McKim *et al.*, 1987a, 1987b). Once an organic 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-Queralto 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 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; Scott *et al.*, 2006). Earlier work indicated that freshwater killifish only drink at a rate approximately 10 % that of fish acclimated to brackish water (approximately 14ppt) (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. 2.5A). 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.2.5B), indicating a different explanation for the appearance of EE2 in the gut, aside from drinking, as outlined below.

EE2 Accumulation in Specific Tissues

Together, the gall bladder and liver accounted for more than 50 % of the total accumulation after 2 h in the reference condition (Fig. 2.4A,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. It is known that lipophilic xenobiotics, as well as other endogenous and exogenous substances that are found circulating in the blood, are metabolized/degraded by hepatic enzymes in the liver. These substances are then secreted in a detoxified 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 indicator of exposure to xenoestrogens (Fenlon *et al.*, 2010; Ruddock *et al.*, 2003). EE2 in the bile has likely been broken down into phase II metabolites (conjugates of glucuronic acid and sulphate) by the liver. These compounds may be transformed 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). Biliary secretion of EE2 or its metabolites 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. Notably, the appearance of a substantial portion of this chemical in the gall bladder after only 2 hours indicates very rapid metabolism. Sampling usually occurs only after chronic exposures (Felon *et al.*, 2010), indicating the gall bladder as the main site of uptake after extended contact. It is unusual for gut depuration of an organic to happen before 24 h, as uptake of the chemical before this can

vary 5-fold (Tyler *et al.*, 2005). Furthermore, the differences between chemical processing and uptake greatly vary between species and it is very hard to characterize how an organic such as EE2 might be depurated out of the body with different species of fish (Tyler *et al.*, 2005). However, we repeatedly found that in the killifish, the largest portion of the accumulation was always in the gall bladder after 2 hours; this is likely attributed to species differentiation in both processing and uptake of this xenoestrogen. Chronic studies would be ideal to determine if this pattern of uptake is similar over longer periods of time.

Furthermore, 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). Once bound to the receptor, EE2 will induce a vitellogenic response in both sexually mature male and female fish (Sumpter and Jobling, 1995; Werner *et al.*, 2003). Once this induction of VTG occurs, there is production of lipid droplets required for VTG formation, thus increasing the lipid content of the liver (Peute *et al.*, 1978; Varanasi and Stein, 1991). Two of the major storage sites for lipids are the liver and red muscle (Peute *et al.*, 1978), so it is not surprising that EE2 accumulates preferentially in this organ.

The carcass accounted for about 30% of EE2 accumulation in killifish, but surprisingly 70 % of the total in trout (Fig 2.4 C, D). These high EE2 tissue burdens could be due to a combination of factors. In nature, rainbow trout are more active swimmers when compared to killifish, referred to as the “mud minnow” because of its resting benthic nature (Burnett *et al.*, 2007). Furthermore, it is hard to compare uptake

rates and processing rates between these two species, when it is known that processing of xenobiotics is very different between species of fish (Tyler *et al.*, 2005). The carcass contains all organs that are 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). Furthermore, the differences in lipid composition and/or distribution between trout and killifish could be responsible for the discrepancy between the EE2 burdens in the carcasses (Dobbs and Williams, 1983; Connell, 1990; Yang *et al.*, 2000). Al-Ansari and colleagues (2010) found a correlation between EE2 and lipid content in the whole body of the short redhorse suckers indicating the higher the lipid composition, the higher the EE2 accumulation.

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 (SWCC's) 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 PVC's (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, SWCC's become prevalent on the gill epithelium (Wood, 2001) and SWCC's 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 gasses and oxygen uptake (Perry, 1997, 1998), and presumably EE2 as well. This may explain the lower EE2 uptake seen in 100 % sea water (32 ppt) versus 50 % sea water (16 ppt).

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 (FWCC's) present in other species of teleosts (Wood and Marshall, 1994; Patrick *et al.*, 1997; Patrick and Wood, 1999; Wood and Laurent, 2003). These cuboidal cells are thick and found intermingled with 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 SWCC's are few in number and lay dormant, covered by pavement and cuboidal cells, thereby increasing gill epithelial thickness (Perry, 1997, 1998; Evans *et al.*, 2005). 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 an increase in EE2 uptake compared to freshwater-adapted killifish but overall lower 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 SWCC's, ultimately increasing gill epithelial thickness to adjust for the change in ionic concentration in the water (Laurent *et*

al., 2006). At 24 h after transfer, these killifish will not have a complete freshwater gill, but will also not retain the typical seawater gill either; this can explain why we have an uptake that is actually in the middle, between our observed freshwater and the 50 % seawater uptakes of EE2.

MacLatchy and colleagues (unpublished data) found a similar trend between vitellogenin production 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 expression of VTG in 50 % sea water than in fresh water. Our study correlates with this observation, as there was a higher uptake of EE2 at a 50 % salinity, which would likely contribute to the higher expression of VTG under similar conditions.

In contrast to the killifish, salinity (fresh water versus 16 ppt) had no effect on EE2 uptake in rainbow trout. Rainbow trout have the normal FWCC's displayed in fresh water (Laurent and Perry, 1990), and the cuboidal cells in killifish are fundamentally different from the FWCC's present in trout (Laurent *et al.*, 2006). The bioaccumulation obtained in the rainbow trout is likely different due to different cellular morphologies present in the gills, resulting in increased accumulation in this species relative to the killifish (Fig 2.3A, 2.3B).

Relevance of the Findings

The experimental evidence of this chapter suggests that it may be possible to develop a model to predict bioavailability and bioaccumulation of organic endocrine disrupting substances as a function of environmental variables. Any environmental condition which 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. 2.6). The influence of salinity may be to change the slope or intercept of the relationship. 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. A future goal will be to incorporate the role of dissolved oxygen variations in such a model.

CHAPTER 3: GENERAL SUMMARY AND CONCLUSIONS

In this study, we demonstrated that changes in the aquatic environment have the potential to ultimately affect the uptake of a potent endocrine disrupter, ethynylestradiol. Temperature and salinity exerted large effects on the uptake rate of EE2 in the killifish, effects which were likely related to events at the gill epithelium. Drinking seemed to make a negligible contribution to the uptake under different salinity conditions, and little was taken up through the skin of recently killed animals, thus implicating the entry point of EE2 uptake as the gills. We tested four experimental temperatures (4, 10, 18, 26°C) under conditions of constant salinity (16 ppt) and three different salinities (0, 50, 100 %) at 18°C. Overall increases in temperature resulted in increases in uptake of EE2 in killifish. Furthermore, changes in salinity resulted in an increase in uptake rate from fresh water to 50 % sea water, and then a decrease from 50 % sea water to 100 % sea water. When temperature and salinity changes were applied, they altered both the uptake rate of EE2 and the oxygen consumption rate concomitantly, supporting our theory that these two molecules share a similar pathway (Figs 2.1-2.5, Chapter 2).

The metabolic rate of poikilotherms is largely impacted by aquatic temperature fluctuations, ultimately changing the demand for oxygen to compensate (Hazel and Prosser, 1974). Since metabolic rate drives oxygen demand, and metabolic rate will parallel changes in temperature, the respiratory epithelium must obtain more oxygen at higher temperatures, while increasing cardiac and ventilator performance to keep up with this requirement (Randall, 1982). Therefore, the gill epithelium and thus changes to it

have been implicated as the rate limiting step in the uptake of any xenobiotic into an aquatic organism (McKim, 1991; Brauner *et al.*, 1994; Yang and Randall, 1995; Yang *et al.*, 2000).

Salinity, however, did not display as clear a trend with the uptake of EE2 and oxygen consumption as the one observed in our temperature series. It is known that there is a respiratory compromise in saline environments by the alteration of cell types (Morgan and Iwama, 1991). We speculate, but have not proven, that alterations in gill morphology due to salinity changes influence the water-to-blood diffusion distance across the gill epithelium so as to directly impede or increase the uptake of oxygen or EE2. While salinity had a biphasic influence on the uptake rate of EE2 and oxygen consumption (least in fresh water, highest in 50 % sea water, intermediate in 100 % sea water) in the killifish, there was no difference in the uptake of EE2 or the oxygen consumption in rainbow trout under two different salinities (fresh water and 50 % sea water). This is likely due to morphological differences of the ionoregulatory cells in the gills between the two species. Killifish adapted to fresh water had the lowest EE2 uptake, likely due to the type of mitochondria rich cells present in the gill epithelium of freshwater adapted killifish; these have been identified as cuboidal cells (Laurent *et al.*, 2006). However, all oxygen and EE2 uptakes were consistent suggesting that uptake pathways for both oxygen and EE2 were altered in different salinities similarly. We hypothesize that the differences observed with salinity are due to different cell types present, increasing or decreasing the diffusion distance for both these molecules.

The distribution of EE2 accumulation amongst different tissues was consistent among killifish in all series, implicating the gall bladder and liver as the main sites of accumulation after 2 hours, followed by the gut and carcass. This may be due to the physiological functions that each of these organs plays. The liver has many roles, including lipid synthesis, estrogen reception and detoxification of toxicants (Peute *et al.*, 1978; Varanasi and Stein, 1991; Forlin *et al.*, 1995; Sumpter and Jobling, 1995; Blom *et al.*, 2000; Tollefsen *et al.*, 2002; Werner *et al.*, 2003). The gall bladder receives these detoxified chemicals and delivers them to the gut (Grosell, 2000); therefore, it is not surprising to see the largest uptake in these organs. Furthermore, drinking rate made little contribution to the uptake of EE2, implicating the role of the gall bladder as the main contributor to gut accumulation. The carcass role in accumulation of EE2 was somewhat unclear due to the fact that it encompassed many organs not separated. However, we have hypothesized that adipose tissue left in the carcass and non-excised organs which contain estrogen receptors are the probable sites where EE2 is partitioning (Tyler *et al.*, 1998). There were differences in the relative accumulation of EE2 in the carcass between killifish and rainbow trout. This was hypothesized to be attributed to differences in adipose tissue between the two species.

Future Directions

The goal, ultimately, is to produce a model for uptake of EE2 under changing environmental conditions reflecting that of an estuarine, tidal environment where both temperature and salinity fluctuate diurnally and seasonally. To fully complete this goal, it

would be valuable to investigate conditions of both low and high levels of dissolved oxygen, as well as exercise. Like temperature, low partial pressures of oxygen in the water will cause fish to increase ventilation rate and increase the number of lamellae being perfused (Randall and Daxboeck, 1984). Hyperoxia causes the opposite effect (Wood and Jackson, 1980). However, neither of these treatments will likely increase or decrease oxygen uptake rate, but rather serve to maintain it at a fairly constant level. Exercise will likely increase oxygen consumption rate, and cause other fundamental changes in the physiology of the fish, such as altered blood flow distribution (Farrell *et al.*, 2001; Thorarensen and Farrell, 2006). Will the relationship between EE2 uptake rate and oxygen consumption still hold under these conditions? Furthermore, investigating EE2 uptake under chronic exposures may be more environmentally realistic. Will uptake rate change with the duration of exposure? Will the tissue-specific sites of uptake change over time? Determining the answers to these questions would help to complete our predictive model for EE2 uptake in a typical estuarine environment. These experiments are currently under way.

Since there appeared to be a complex relationship between salinity and uptake and we have speculated about cell rearrangements on the branchial epithelium affecting the uptake rate of EE2, it would be of great importance to actually investigate gill morphology as the killifish goes through salinity changes. For example, using a combination of light (LM), transmission (TEM), and scanning electron microscopy (SEM), it would be very useful for identifying the types of cells present on the gill epithelium as the killifish go through a transfer from intermediate salinity to 100 % sea

water or fresh water for approximately one week, the time needed to create a full freshwater or seawater gill (Wood and Laurent, 2003). Indeed, it would also be critical to measure the water-to-blood diffusion distance under salinity transfers to provide evidence that in fact the diffusion distance increases or decreases with changing salinity.

Overall, the aim of this study was to investigate the rate of uptake of 17- α -ethynylestradiol (EE2) – a potent endocrine disrupting substance – in *Fundulus heteroclitus*, under conditions of varying temperatures and salinities. We have shown that temperature altered EE2 uptake and oxygen consumption, which was likely a result of changing metabolic rate in killifish. Moreover, salinity had varying effects on EE2 uptake and oxygen consumption with 50 % seawater inducing the highest EE2 uptake and oxygen consumption. These responses may be species-specific as there was no change in EE2 uptake or oxygen consumption in response to salinity in rainbow trout. Overall, a relationship was established between oxygen consumption and EE2 uptake; however, further studies are needed to fully explore this relationship. Indeed, changing any environmental parameter which alters oxygen consumption will likely also impact EE2 uptake because the route of entry is suspected to be similar at the gills.

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