The Effects of 17α-Ethinylestradiol (EE2) and Hydroxypropyl-β-Cyclodextrin (HPβCD) on the Heart Rate and Metabolism of Embryonic Japanese Medaka (*Oryzias latipes*)

By

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An oral defense of this thesis took place on April 11, 2019 in front of the following examining committee:

Examining Committee:

Chair of Examining Committee	Dr. Janice Strap
Research Supervisor	Dr. Douglas Holdway
Examining Committee Member	Dr. Andrea Kirkwood
Examining Committee Member	Dr. Sean Forrester
University Examiner	Dr. Julia Green-Johnson
External Examiner	Dr. Mark McMaster, Environment and Climate Change Canada

The above committee determined that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate during an oral examination. A signed copy of the Certificate of Approval is available from the School of Graduate and Postdoctoral Studies.

ABSTRACT

Estrogen toxicity has been an area of priority in aquatic toxicology over the last 20 years. The toxicity of a known estrogen mimic, 17α-ethinylestradiol (EE2), has been attributed to classical estrogen signaling within target and non-target organisms. Recent evidence has indicated that a rapid, non-genomic, non-classical estrogen signaling pathway also exists via the G protein coupled estrogen receptor (GPER). GPER is expressed ubiquitously and has many biological functions, including cardiovascular and metabolic function. Understanding the role of GPER at environmentally relevant concentrations of estrogens could aid addressing many knowledge gaps associated with estrogen toxicity in aquatic environments.

This thesis investigated the effects of EE2 on heart rate and metabolism, as well as EE2 uptake, and elimination in embryonic Japanese medaka (*Oryzias latipes*). Bradycardia (reduced heart rate) was observed in embryos exposed to 10 ng/L of EE2. It was demonstrated that these effects were the result of GPER activation and not estrogen receptor (ER) α and β. A mixture of EE2 and hydroxypropyl-β-cyclodextrin (HPβCD) was also investigated. HPβCD is a commonly used odour suppressant and excipient with the ability to include guest compounds. It was determined that the observed EE2 induced bradycardia was reduced with a 4:1 HPβCD : EE2 molar ratio. Uptake of ^{14}C-EE2 followed a sigmoidal pattern, and chorion permeability increased as development progressed. Elimination of ^{14}C-EE2, showed a pattern of exponential decay following exposure from 6-48 hours post fertilization. HPβCD did not impede uptake of 14C-EE2 across the chorion, suggesting that HPβCD may cross the chorion. Uptake of the HPβCD – EE2 mixture into the tissues of the developing embryo should be investigated. A conclusive link was not

determined between EE2 induced bradycardia and embryonic oxygen consumption (metabolic rate). The absence of metabolic effects might be mitigated by cutaneous gas exchange by embryonic fish. This data suggests that embryonic heart rate may not be an ideal measure of metabolic rate in embryonic medaka.

This thesis is valuable to the field of aquatic toxicology as it highlights GPER as a novel mechanism of action for EE2 toxicity as well as the role of HPβCD in mixture toxicity. This work and future research into the role of GPER will aid in the overall understanding of estrogen toxicity to fish.

AUTHOR'S DECLARATION

I hereby declare that this thesis consists of original work of which I have authored. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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The research work in this thesis that was performed in compliance with the regulations of UOIT's Animal Care Committee under **AUP 14-002**.

Jordan Christopher Anderson

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STATEMENT OF CONTRIBUTIONS

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication. I have used standard referencing practices to acknowledge ideas, research techniques, or other materials that belong to others. Furthermore, I hereby certify that I am the sole source of the creative works and/or inventive knowledge described in this thesis.

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LIST OF ABBREVIATIONS

µCi – Microcurie

µl – Micro liters

µM – Micro molar

11-KT – 11 ketotestosterone

17,20-DHP – 17α,20β -dihydroxypregn-4-en-3-one

AOPs – Adverse outcome pathways

ANOVA – Analysis of Variance

cAMP – Cyclic adenosine monophosphate

CC – Carrier control

CCAC – Canadian Council on Animal Care

cm – Centimeters

CPM – Counts per minute

DPM – Dissociations per minute

E2 – Estradiol

EE2 – 17α-ethinylestradiol

FLFII – Female leucophore free

FSH – Follicle stimulating Hormone

g – Grams

GnRH – Gonadotropin releasing hormone

GPCR – G protein-coupled receptor

GPER – G protein-coupled estrogen receptor

GDP – Guanosine diphosphate

GtH1 – Gonadotropin 1 GtH1

GtH2 – Gonadotropin 2 GtH2

GTP – Guanosine triphosphate

GSI – Gonadosomatic index

hpf – Hours post fertilization

HPG – Hypothalamus pituitary gonadal axis

HPGL – Hypothalamus pituitary gonadal liver axis

HPβCD – Hydroxypropyl-β-cyclodextrin

IPA – Instrument Performance Assessment

kDa – Kilodalton

L – Liters

LH – Luteinizing hormone

LSI – Liver somatic index

mg – Milligram

ml – Millilitre

MO_2 – Oxygen Consumption Rate

mRNA – Messenger ribonucleic acid

mV – Millivolts

ng – Nanogram

NOEC – No-observed-effect-concentration

PPCP – Pharmaceuticals and personal care products

rpm – Rotations per minute

SE – Standard error

SNC – Self-Normalizing Calibration

T3 – Triiodothyronine

tSIE – Transformed Spectral Index of the External Standard

VTG – Vitellogenin

v/v – Volume / Volume

WWTP – Waste water treatment plant

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Chapter 1: Literature Review

1. Chapter 1: Literature Review

1.1. Introduction

Over the last 20 years, there has been heightened environmental awareness regarding the release of pharmaceuticals and personal care products (PPCPs) into the aquatic environment. Pharmaceuticals are a diverse group of biologically active medicinal compounds used to treat disease in both humans and livestock (Corcoran *et al.*, 2010), whereas personal care products such soaps, detergents, and deodourizers are used on a daily basis for personal hygiene. High levels of consumption has resulted in the detection of PPCPs in surface waters in the ng/L to µg/L range. PPCPs at these concentrations have been shown to have the ability to elicit affects in non-target organisms (Overturf *et al.*, 2015).

Steroid hormones are among the most commonly studied pharmaceuticals in environmental toxicology. These pharmaceuticals are very important due to their ability to elicit toxicological responses to non-target organisms (Parrott and Blunt, 2005; Kidd *et al.*, 2007; Overturf *et al.*, 2015). Synthetic steroids in human contraceptives are of particular interest because of their frequency of prescription, presence in the environment, and their ability to cause physiological impacts in non-target organisms at environmentally relevant concentrations (Parrott and Blunt, 2005; Kidd *et al.*, 2007; Santos *et al.*, 2014). The most heavily studied synthetic steroid in the field of environmental toxicology is 17α-ethinylestradiol (EE2). Many studies have shown that EE2 can cause reproductive impairment in fish at concentrations in the low ng/L range (Parrott and Blunt, 2005; Kidd *et al.*, 2007; Overturf *et al.*, 2015). The focus of much of this research has been centered

on the effects of EE2 activating nuclear estrogen receptors in fish. These receptors are ligand-dependent transcription factors, and when activated often result in modulation of gene expression. Changes in gene expression from EE2 exposure have been shown to alter protein production, contributing to the feminization and reproductive impairment in fish (Parrott and Blunt, 2005; Kidd *et al.*, 2007).

Currently, there is a significant knowledge gap in the role of G protein-coupled estrogen receptor (GPER), formerly known as GPR30, in EE2 toxicity (Diamante *et al.*, 2017). Activation of this receptor is typically thought to result in a more rapid physiological response since it is not a ligand-dependent transcription factor (Diamante *et al.*, 2017; Romano *et al.*, 2017). Recent research has suggested that embryonic heart rate can be utilized as an important physiological biomarker to assess GPER activity in fish (Romano *et al.*, 2017). The impacts of EE2 on fish embryonic heart rates in and metabolism at environmentally relevant concentrations have not been studied in detail. One objective of this thesis is therefore to assess the effects of EE2 on heart rate and embryonic metabolism in relation to GPER using embryonic Japanese medaka (*Oryzias latipes*).

Another interesting group of PPCPs is deodourizers, specifically cyclodextrins. Cyclodextrins are commonly used in both the personal care and pharmaceutical industry as deodourizers and as excipients, respectively. This is done through the inclusion of guest compounds within the cavity of the cyclodextrin molecule (Del Valle, 2004). Inclusion of guest compounds often results in improved solubility and altered bioavailability. In the field of environmental toxicology, the potential for cyclodextrins to alter the toxicity of the compounds that they include has been understudied. Unfortunately, effluent and surface water concentrations of cyclodextrins are unknown, highlighting a future area of research.

However, understanding the effects of toxicants in the presence of cyclodextrins is an important step in characterizing whole-effluent toxicity to non-target organisms. Another objective of this thesis is thus to determine the effects of cyclodextrins on EE2 toxicity.

1.2. Aquatic Toxicology

Aquatic toxicology is the study of the toxicity of contaminant substances on aquatic organisms, of which fish are often utilized due to their economic importance, and their continuous surface water exposure via effluent and run-off.

There are two major classes of toxicity, acute and chronic. Acute toxicity is defined as a stimulus that is severe enough to cause a response within a short period of time, typically 96 hours for fish (Sprague, 1969). Chronic toxicity is a measure of a biological response to a stimulus over a prolonged period. A chronic exposure is usually defined as lasting greater than one tenth of an organism's life cycle (Sprague, 1969).

The majority of compounds studied in a laboratory setting are tested individually. While this approach is crucial for understanding individual modes of action and effect concentrations, it does not represent real world exposures in which organisms encounter multiple chemicals simultaneously.

1.2.1. Mixture Toxicity

Toxicity studies of mixture contaminants have become increasingly more frequent. Mixture toxicity is important when assessing the effects of effluents or surface waters that contain more than one potential toxicant. In brief, there are three major toxicological outcomes when toxicants are mixed: antagonism, synergism, or no interaction (Sprague,

1970). If two or more toxicants mixed together result in a reduction in toxicity, this is known as antagonism (Sprague, 1970). Synergism occurs when the mixture toxicity is additive, more than additive (potentiation), or less than additive (Sprague, 1970). Mixture toxicity is inherently more complexed than individual compound toxicity, but it is an important area of research as it better represents real world conditions.

1.2.2. Embryonic Toxicity Testing

The use of fish embryos in aquatic toxicology studies has increased in recent years. There has been a substantial movement in animal ethics and testing to adhere to the three R's principle (replacement, reduction, and refinement), when utilizing animals for research (Braunbeck *et al.*, 2015). Additionally, the pressure to increase throughput while reducing experimental costs has been problematic in many experimental laboratories. The use of embryonic fish in aquatic toxicological studies is one approach to help alleviate this issue.

Embryonic fish are thought to be far less sentient than juvenile or adult fish. Thus, in most animal ethics regulations, embryo fish are not included as 'animals' which provides for the replacement portion of the three R's. Additionally, the number of adults or juvenile fish required for testing is reduced through the use of embryos. Embryonic testing can allow for greater refinement of concentration ranges or aid in identifying lethal compounds without having to expose juvenile or adult fish to these levels of toxicants. Embryonic toxicity testing can also improve throughput while reducing associated costs. For example, the volume of exposure solution is dramatically lower, often 1000-fold, when exposing embryos compared to adults, saving on chemical costs, waste management as well as laboratory space. Embryonic testing retains the benefit of being able to test toxicity during

the major stages of development including organogenesis. This may allow for a better understanding of the mode of action of a particular toxicant. Finally, embryonic development of warm water fish is often relatively quick, between 4 and 10 days post fertilization, which coupled with the reduced volumes required, aids in the throughput of testing.

Embryonic testing does have some limitations. Firstly, the chorion surrounding the embryo has the potential to block the uptake of some toxicants (González-Doncel *et al.*, 2004; Braunbeck *et al.*, 2005). Generally, toxicants that are hydrophilic or bulky in size have more difficulty crossing the chorion (Braunbeck *et al.*, 2005). This could potentially result in no-effect-concentrations (NOECs) in embryos that would otherwise result in toxicity to juveniles or adults. On the other hand, embryonic fish have a very high surface area to volume ratio, potentially increasing the relative uptake of toxicants that are able to readily cross the chorion. Researchers must thus be aware of the chemical nature of the toxicant(s) being used. Furthermore, organismal development is often not completed during embryogenesis and hatching. Many embryonic physiological processes may thus not be fully functional compared to adults and juveniles, which can impact embryonic susceptibility to a particular toxicant. For example, a toxicant that must be bio-transformed to elicit a toxic response may be perceived as non-toxic in an embryo that does not yet have the capabilities to conjugate toxicants.

The Organization for Economic Co-operation and Development (OECD) has created guidelines for toxicant testing using aquatic organisms. Recently Test No. 236, Fish Embryo Acute Toxicity Test (FET) was developed (Braunbeck *et al.*, 2015). This test was developed to utilize zebrafish (*Danio rerio*) over 96 hours. A strong correlation between

the FET and traditional acute toxicity tests was shown, suggesting that FET is useful for assessing acute toxicity at later life stages (Braunbeck *et al.*, 2015).

1.3. Test Species

1.3.1. Teleost Fish as Research Organisms

Fish are among the most commonly used research organisms when assessing aquatic toxicity. This is partly due to their high economic, intrinsic, and ecological value in Canada and globally. Fish also inhabit surface waters, which are a major receiving environments for many pollutants as they are washed down drains or run off of the landscape (Figure 1).

Teleost fish are specifically seen as important research organisms in the field of aquatic toxicology due to the proportion of species they represent globally. They are the largest infraclass in the class Actinopterygii, and represent 96 % of all extant species of fish (~26000 species), and 40 % of all vertebrate species globally.

Teleost fish are particularly valuable when assessing the toxicity of pharmaceuticals because they exhibit a high level of biological conservation for many human drug targets. In one study using 10 species of fish, including Japanese medaka, fish showed a high level of conservation in 81-86 % of the of 459 human drug targets examined (Brown *et al.*, 2014). Additionally, a strong conservation in six nuclear steroid receptor ligand binding domains was also observed (Brown *et al.*, 2014). The global representation of teleost fish coupled with the likelihood of exposure and potential susceptibility to environmental contaminants make fish valuable research organisms.

1.3.2. Japanese Medaka (*Oryzias latipes*)

The teleost species of fish used in this thesis was the Japanese medaka (*Oryzias latipes*), which are native to freshwaters in China, Korea, and Japan. Medaka can reach sexual maturity in approximately 2 months with a maximal size of 2.5 – 3 cm and exhibit sexually dimorphic dorsal fins at maturity (Shima and Mitani, 2004). Medaka are oviparous, dawn spawners, and fertilize externally, often producing 10-30 eggs per female per day (Iwamatsu, 2004). These factors and their ease of maintenance have made medaka excellent experimental organisms. Medaka embryos have a clear chorion, allowing for direct observation of the embryonic stages of development (Iwamatsu, 2004; Shima and Mitani, 2004). Consequently, medaka have been used extensively in reproductive and embryo toxicity studies.

Wild type medaka have leucophores that emit auto-fluorescence when observed under a fluorescence microscope (Shima and Mitani, 2004). However, many different strains of the Japanese medaka exist, including the female leucophore-free (FLFII) strain. FLFII medaka utilized in this thesis were obtained from the National Institute for Basic Biology (*Okazaki, Japan*) and cultured in-house. FLFII females do not produce leucophore cells, while males remain as wild type (Balch *et al.*, 2004). This strain thus allows gender differentiation as early as 72 hours post fertilization in FLFII medaka. This is important for assessing gender differences in toxicity during embryogenesis which is often not possible using other species of fish. Importantly, no differences in E2 sensitivity were observed between FLFII and wild type medaka gonadal phenotypes (Balch *et al.*, 2004).

1.4. Teleost Physiology

1.4.1. Embryogenesis

The embryonic phase of a fish is defined as the period of time after fertilization and before hatching. The embryo is encapsulated within an envelope known as the chorion and remains dependent on the yolk for nutrients. Embryonic development, or embryogenesis of fish can be broken down into 6 broad periods: fertilization, cleavage, germ layer formation, axis formation, neurulation, and somite development. The following paragraphs briefly outline some of the 39 stages of normal embryonic Japanese medaka development as described by Iwamatsu (2004).

Stage 0 of medaka development consists of an unfertilized mature egg, they are spherical with an average diameter of 1200 µm. Unfertilized eggs contain yolk within the chorion and attachment fibers on the exterior surface. Stage 1 is initiated when an egg is stimulated by the sperm of a male medaka. This occurs externally and initiates a wave of exocytosis, the chorion then hardens. Male and female pronuclei fuse together forming the zygote nucleus, concluding stage 2.

Cleavage begins to take place at approximately 1 hour post fertilization and is classified by the division into 2 cells. The cells continue to cleave in stages 2 to 8, during this phase the number of cells doubles with each cleavage and the volume of each cell halves. The embryo then will begin to enter germ layer formation.

Germ layer formation is characterized by the formation of the endoderm, mesoderm, and the ectoderm. In medaka this occurs at approximately 4 hours post fertilization the embryo during stages 8 to 16, the morula, blastula, and gastrula stage.

During these stages blastodermal cells begin to grow flattened to the curvature of the yolk until it is nearly enveloped.

At 25 hours post fertilization the embryo enters the neurula stage, stages 18 and 19. The head of the medaka begins to form followed by the brain, nerve cord, and optic bud. This leads into the somite stage of development.

The somite stage is defined as the division of the embryo into defined segments and begins at 27.5 hours post fertilization. The eyes and auditory vesicles begin to form, and at 38 hours post fertilization the tubular heart becomes present underneath the head (stage 22). The heart begins to beat at stage 24, 44 hours post fertilization, and pulsation of the heart extends to the forebrain. Circulation begins at 50 hours post fertilization with a heart rate between 70 and 80 beats per minute. During stage 28, 64 hours post fertilization the bulge of the liver becomes definitive. In the heart, the sinus venosus, atrium, ventricle and bulbous arteriosus differentiated in stage 29, 74 hours poster fertilization. The gills and brain become visible, and the hepatic vein is functional at 82 hours post fertilization. Stage 36 known as the heart development stage is initiated at 144 hour post fertilization. The atrium and ventricle lie adjacent to one another in the lateral view. At 168 hours post fertilization the pericardial cavity visibly surrounds the heart. Finally, at stage 39, at 216 hours post fertilization, the embryo begins to hatch. Medaka embryos hatch by dissolving the inner chorion and escape tail first (Iwamatsu, 2004).

1.4.2. The Chorion

The chorion, is an acellular envelope that separates ovulating eggs and developing embryos from the surrounding environment (Hart, 1983). The major functions of the

chorion are to aid in fertilization, gas exchange, attachment to substrates in demersal eggs, as well as protection from microorganisms, physical harm from toxins or toxicants (Schoots, 1982). Most teleost fish possess a multi-layered chorion. The outer surface is commonly coated in filaments which aid in attachment to other eggs or substrates (Schoots, 1982; Iwamatsu, 2004). A primary envelope is often located below the outer surface. Depending on the species, a secondary or tertiary envelope may also exist.

1.4.2.1. Chorion Permeability

A major assumption when testing the toxicity of a substance is that the chemical in question is able to interact with the test organism. This can be problematic when assessing embryonic toxicity. As with many biological membranes, bulky or hydrophobic compounds have difficulty crossing the chorion while water, electrolytes, and small lipophilic compounds cross the chorion relatively freely (Braunbeck *et al.*, 2005). The layers of the chorion are spanned by toroidal shaped pore canals that have been shown to restrict entry based on the size and charge of the molecule (Kais *et al.*, 2013). Zebrafish pore canals have been known to exclude compounds greater than 3-4 kDa (Souder and Gorelick, 2017). The permeability of compounds often varies across embryogenesis and species (González-Doncel *et al.*, 2004).

After fertilization, and in the presence of water, the chorion begins to undergo hardening. Chorion hardening is characterized by a dramatic increase in the rigidity of the membrane caused by a change in the protein composition. During this stage, the permeability of the chorion often decreases; as the embryo develops, permeability often increases over time (Adams *et al.*, 2005). In carp (*Cyprinus carpio*), as development

progresses, pore diameter increases, often improving permeability (Rubstov, 1981). Japanese medaka embryos begin to degrade their chorion at stage 34, approximately 120 hours post fertilization, increasing chemical permeability (González-Doncel *et al.*, 2004).

1.4.3. Teleost Cardiovascular System

The cardiovascular system of fish is similar to most vertebrates in that it is a closed system that is responsible for delivering blood to tissues for gas and nutrient exchange. Teleost fish have a single circuit cardiovascular system that is pressurized by the pumping of a three chambered heart consisting of the sinus venosus, atrium and ventricle (Evans *et al.*, 2013). Deoxygenated blood enters the heart through the sinus venosus, which acts as a thin walled reservoir. From there, the blood travels to the thicker walled atrium which can expand greatly when filled with blood. As the atrium contracts, the atrioventricular valve opens allowing blood to flow into the relaxed ventricle (Evans *et al.*, 2013). The ventricle is thick walled and its contraction is responsible for producing the bulk of the blood pressure in fish. Blood exits the heart through the outflow tract. The outflow tract is an elastic structure that mediates the connection between the ventricle and ventral aorta by absorbing some of the high pressure released from the ventricle (Evans *et al.*, 2013). Blood then enters the gills where it is oxygenated through a counter current mechanism of gas exchange before traveling to the rest of the body. After gas and nutrient exchange in the body, the deoxygenated blood enters the sinus venosus, completing the circuit (Evans *et al.*, 2013).

1.4.3.1. Teleost Heart Rate

Cardiac control systems adjust heart rate in response to changes in respiratory demands, or metabolic rate (Vornanen, 2016). In most teleost fish, cardiac control acts on pacemaker cells located in specialized tissue at the junction of the sinus venosus and atrium (Evans *et al.*, 2013; Vornanen, 2016). Pacemaker cells control the rate of cardiac myocyte contraction through the initiation of action potentials in vertebrates (Vornanen, 2016). Cardiac myocytes located in the atrium and ventricle start and finish an action potential at a stable negative resting membrane potential, usually between -70 and – 90 mV (Vornanen, 2016). An action potential in the cardiac myocyte is initiated by an action potential from pacemaker cells; this causes the rapid depolarization of the membrane to +10 to +40 mV (Vornanen, 2016). This change is the result of the opening of sodium channels allowing for the influx of Na^+ ions. Once at the peak of the action potential, repolarization is initiated by the opening of potassium channels. This results in the efflux of K^+ ion from the cardiac myocytes causing the membrane potential to become more negative (Vornanen, 2016). However, a simultaneous influx of Ca^{2+} ions during this phase delays the repolarization causing a prolonged positive plateau in the action potential, especially in ventricular myocytes (Vornanen, 2016). This calcium induced plateau is essential to prevent cardiac muscles from contracting prematurely (Vornanen, 2016). The plateau also extends the contraction of ventricular myocytes allowing for the ejection of blood to take place (Vornanen, 2016).

Pacemaker action potentials differ from atrium and ventricle action potentials in that they do not have a stable resting membrane potential. The resting membrane potential of pacemaker cells is usually between – 50 and -65 mV and they experience creeping

membrane depolarization (Vornanen, 2016). This creeping membrane depolarization results in a regular firing of the pacemaker action potential at the threshold potential (Vornanen, 2016). The plateau phase of pacemaker action potentials is not as prominent as in the ventricular and atrial myocytes due to the rapid repolarization caused by K^+ efflux.

1.4.3.2. MO_2 and Heart Rate

The rate of oxygen consumption (MO_2) by an organism is a useful method for assessing metabolic rate. MO_2 is often measured using swim tunnels or microplate respirometers. Due to the cost of these instruments, as well as the difficulty in measuring MO_2, particularly during the embryonic stage, heart rate has been used as a surrogate measurement for metabolic rate.. A study by Folkerts *et al.,* (2017), demonstrated that hydraulic fracturing flow-back and produce water caused a decrease in larval zebrafish heart rate. Using a Loligo® Microplate respirometer the authors also measured a decrease in MO_2 of exposed larvae. This use of a respirometer in conjunction with monitoring heart rate indicates that an alteration in heart rate can correspond to a change in MO_2. However, heart rate is still not a direct measurement of metabolism, and may be influenced by other factors. Fish are ectotherms and temperature can play a significant role in metabolic rate which must be controlled for (Vornanen, 2016).

1.4.4. Estrogens

1.4.4.1. 17β-estradiol (E2)

In vertebrates, steroid hormones are derived from cholesterol and are an important component of the endocrine system. In female fish, 17β-estradiol (E2) is a major reproductive steroid. E2 is known to influence behaviour, gonadal differentiation, and secondary sexual characteristics. The chemical properties of E2 are outlined in Table 2. A common biomarker for estrogen exposure in fish is induction of vitellogenin (VTG) in males (Kime, 1999). VTG is a yolk precursor protein normally only expressed in females and it is important in oocyte production (Kime, 1999).

1.4.4.2. 17β-estradiol Regulation

Reproductive hormones are primarily regulated through the hypothalamus-pituitary-gonadal (HPG) axis in mammals, and the hypothalamus-pituitary-gonadal-liver (HPGL) axis in oviparous organisms (Arukwe and Goksøyr, 2003). In fish, gonadotropin releasing hormone (GnRH) is released from the hypothalamus, acting on the pituitary gland resulting in the release of gonadotropin 1(GtH1) and Gonadotropin 2 GtH2 (Arukwe and Goksøyr, 2003). Through plasma transport,GtH1 and GtH2 activate a G-protein mediated signal transduction within the gonads, inducing steroidogenesis in fish (Clark *et al.*, 1994; Nakamoto *et al.*, 2012). In excess, steroids will activate a negative feedback loop beginning in the hypothalamus and pituitary. This interruption inhibits the release of GnRH, interrupting the HPGL axis, thereby interrupting steroidogenesis and reducing the production of E2 in the ovaries (Kime, 1999; Arukwe and Goksøyr, 2003).

1.4.5. Estrogen Receptors

Estrogen receptors are a group of proteins that utilize 17β-estradiol as a ligand for activation. There are two classes of estrogen receptors, nuclear estrogen receptors and membrane estrogen receptors.

1.4.5.1. Nuclear Estrogen Receptors

There are two nuclear estrogen receptors ERα and ERβ, both are ligand-dependent transcription factors and their activation often results in alterations in gene transcription (Delaunay *et al.*, 2000; Romano *et al.*, 2017). In brief, when ERα or ERβ are activated they homodimerize and interact with estrogen response elements located on target genes leading to alterations in gene transcription (Delaunay *et al.*, 2000). This is often referred to as classical estrogen receptor signaling (Prossnitz and Barton, 2014). The mechanism of toxicity of EE2 has been focused on this classical estrogen receptor signaling (Jayasinghe and Volz, 2012).

1.4.5.2. Membrane Estrogen Receptors

Membrane estrogen receptors differ in that they are G protein-coupled receptors (GPCR) and are not ligand-dependent transcription factors. GPCRs are a group of receptors that when activated by many signaling molecules, such as hormones or neurotransmitters, initiate various rapid cellular responses (Jayasinghe and Volz, 2012). GPCRs consist of a seven transmembrane receptor coupled to a g-protein. g-proteins are made up of three subunits, α, β, and γ, the α and γ subunits are anchored into the plasma membrane (Trzaskowski *et al.*, 2012). When inactivated the GPCR is bound to guanosine diphosphate

(GDP) which is also bound to the α-subunit of the g-protein. Upon activation by a ligand, a conformational change will occur, resulting in the α-subunit binding to guanosine triphosphate (GTP) instead of GDP resulting in the dissociation from the receptor and the β – γ dimer (Trzaskowski *et al.*, 2012). Depending on the GPCR, the α-subunit, and the β – γ dimer will travel along the membrane altering other proteins (Trzaskowski *et al.*, 2012). A prominent GPCR, and the receptor of interest for this research project is the G protein-coupled estrogen receptor (GPER), formerly GPR30.

1.4.5.2.1. G Protein-Coupled Estrogen Receptor (GPER)

GPER is located in the membrane of the endoplasmic reticulum and is thought to be responsible for the some of the rapid non-genomic effects that E2 has on cells (Diamante *et al.*, 2017). GPER activation leads to adenylyl cyclase stimulation and has been linked to increased levels of the secondary messengers, cyclic adenosine monophosphate (cAMP) and Ca^{2+} (Lin *et al.*, 2013; Diamante *et al.*, 2017). The mRNA expression of GPER (*gper1*) occurs throughout the body of vertebrates with higher levels found in the gonad, nervous system, and vasculature (Jayasinghe and Volz, 2012). GPER has been shown to play a significant role in the cardiovascular system of vertebrates (Jayasinghe and Volz, 2012; Diamante *et al.*, 2017; Romano *et al.*, 2017). In mice, GPER has been tied to ventricular hypertrophy, regulation of blood pressure and vascular tone, and atherosclerosis progression (Jayasinghe and Volz, 2012).

One of the challenges in studying the role of GPER is differentiating the effects of GPER from the effects of classical nuclear estrogen receptor activation (Romano *et al.*, 2017). Romano *et al.*, (2017) demonstrated the independence of GPER using embryonic

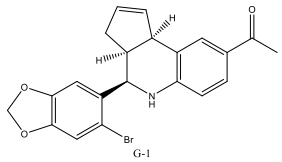
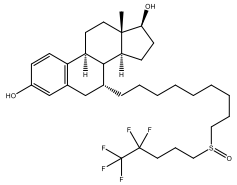
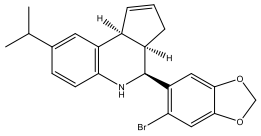
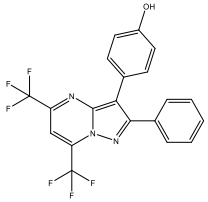
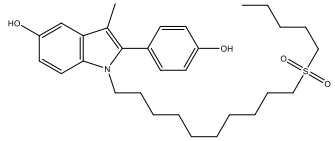
zebrafish. They observed that E2 can significantly increase embryonic heart rate of zebrafish at 3.67µM (1 mg/L). They used specific agonists and antagonists for GPER and nuclear estrogen receptors to understand the mode of action and determined that GPER activation, not membrane nuclear estrogen receptors, acutely regulated embryonic heart rate in zebrafish (Romano *et al.,* 2017). This finding was confirmed though the use of mutant zebrafish. No change in heart rate was observed in nuclear estrogen receptor loss-of-function embryos while a reduced heart rate occurred in maternal zygote *gper1* knockout zebrafish embryos (Romano *et al.,* 2017). They also found that *gper1* expression regulated thyroid hormone triiodothyronine (T3) in the pituitary; T3 is known to increase heart rate. Exposure of T3 to *gper1* knockout zebrafish rescued the reduced heart rate, while E2 had no effect on these organisms (Romano *et al.*, 2017). This study demonstrated that GPER was able to act as an autonomous estrogen receptor and that T3 acts downstream of GPER to regulate heart rate in embryonic zebrafish (Romano *et al.,* 2017). GPER activation is an understudied mode of action for estrogenic compounds and embryonic heart rate of fish appears to be a valuable endpoint to investigate it.

1.4.5.3. Estrogen Receptor Agonists and Antagonists

Several synthetic agonists and antagonists for both nuclear estrogen receptors and GPER exist. The chemical properties of the estrogen receptor agonists and antagonists are outlined in Table 1. G-1 was first characterized in 2006, and is a known selective GPER agonist displaying strong specificity for GPER and little to no affinity for ERα and ERβ (Bologa *et al.*, 2006). Fulvestrant (ICI) is a known antagonist for ERα and ERβ, as well as an agonist for GPER. G-36 is a selective antagonist for GPER, while PHTPP and ZK

164015 are selective antagonists for ERβ and ERα respectively (Table 1) (Prossnitz and Barton, 2014; Romano *et al.*, 2017).

Table 1: Chemical properties of select estrogen receptor agonists and antagonists, G-1, Fulvestrant, G-36, PHTPP, and ZK 164015.

	G-1	Fulvestrant	G-36	PHTPP	ZK 164015
Estrogen Receptor Activity	Selective GPER Agonist	ERα + ERβ Antagonist / GPER Agonist	Selective GPER Antagonist	Selective ERβ Antagonist	Selective ERα Antagonist
Chemical Structure					
Molecular Formula	$C_{21}H_{18}BrNO_3$	$C_{32}H_{47}F_5O_3S$	$C_{22}H_{22}BrNO_3$	$C_{20}H_{11}F_6N_3O$	$C_{30}H_{43}NO_4S$
Molecular Weight (g/mol)	412.283	606.771	412.320	423.318	513.732
Aqueous Solubility (mg/L at 25° C)	0.8865	6.72	-	0.1112	-
Log K_{OW}	4.901	7.35	6.339	5.055	7.97
pKa	-	-0.88 + 10.32 ǂ		9.832	10.115

Data from: ChemDraw Professional V: 16.0.1.4(77), unless denoted ǂ (https://www.drugbank.ca/)

1.5. Pharmaceuticals and Personal Care Products (PPCPs)

PPCPs represent a very diverse group of chemical compounds used in human and veterinary pharmaceutical industries, as well as cosmetic and personal care products. The pharmaceutical industry has been reported to grow 7% annually, suggesting an increasing influent load at WWTPs (Corcoran *et al.*, 2010; Monteiro and Boxall, 2010). Rising use and environmental presence of PPCPs has led to increased environmental concern.

The major urban sources of PPCPs in the environment are wastewater treatment plants (WWTP) effluents (Yang and Metcalfe, 2006; Corcoran *et al.*, 2010). Pharmaceuticals primarily enter wastewater through urine and fecal elimination by humans and livestock, as well as discarded medication (Rosi-Marshall and Royer, 2012). Personal care products, such as soaps and detergents often enter wastewater when rinsed off after use. Once in wastewater, PPCPs may enter the environment through WWTP effluent (Snyder *et al.*, 2003). A consequence of WWTPs is the increase in effluent volume at a single outflow location. For example the Duffin Creek WWTP in *Ajax Ontario, Canada* serves approximately 1 million people from many surrounding municipalities, discharging into Lake Ontario an average of 340 million liters of treated effluent per day with the capacity to treat 630 million liters per day (Durham Region, 2013). A visual summary of the primary routes of PPCP entry into the environment can be found in Figure 1.

Typically, WWTPs do not have a dedicated step to remove PPCPs which are also deemed to be pseudo-persistent (Rosi-Marshall and Royer, 2012). The continual release of PPCPs allows for the maintenance of surface water concentrations even if the compound is not inherently persistent, resulting in surface water concentrations in the ng/L - µg/L range (Durán-Alvarez *et al.*, 2009). Recent advances in WWTP operational processes,

specifically nitrifying activated sludge, have reduced the effluent estrogenicity (McAdam *et al.*, 2010; Hicks *et al.*, 2017). A WWTPs redox potential, and retention times, hydraulic and solid, has been known to impact the efficiency of estrogen removal (Koh *et al.*, 2008).

The following sections will review two of the PPCPs of interest for this thesis, 17α-ethinylestradiol (EE2) and hydroxypropyl-β-cyclodextrin (HPβCD), with emphasis on the physiological systems that are most affected. These PPCPs were chosen to be studied due to the ability for HPβCD to include EE2, as well as the potential to determine the effects of EE2 on embryonic heart rate potentially through GPER activation.

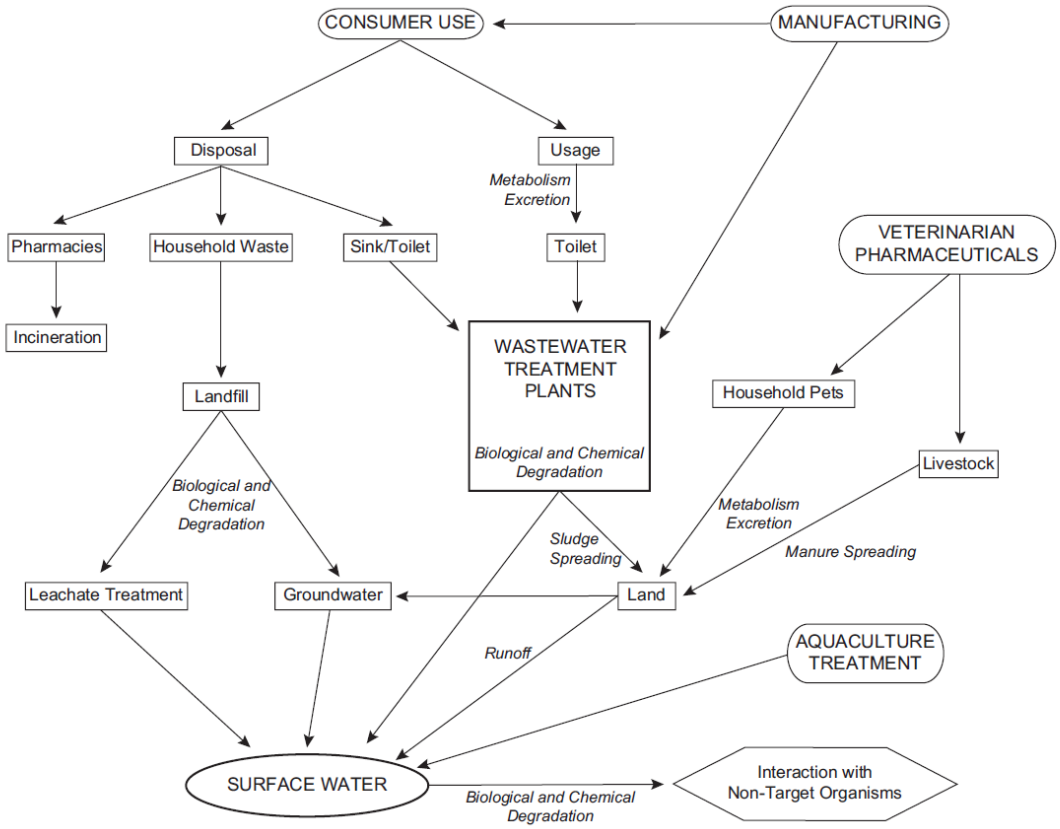


Figure 1: Primary routes of pharmaceuticals and personal care products (PPCPs) into the environment (image from Overturf *et al.*, 2015).

1.6. PPCPs of Interest

The following sections will outline the chemical properties, biological activity and toxicity of EE2 and HPβCD. EE2 was chosen based on its ubiquitous presence in the environment, and its ability to induce a biological response. Although the environmental concentration of HPβCD is currently unknown, it was selected due to its high prevalence in detergents and odour suppressants, and its ability to interact with EE2 (Tang *et al.*, 2018).

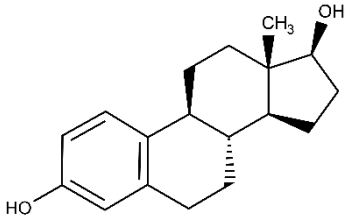
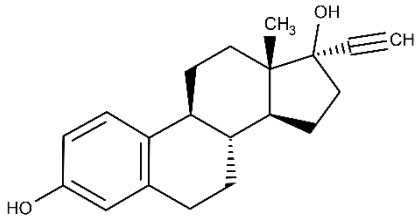
1.6.1. 17α-Ethinylestradiol (EE2)

EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol) is a nonpolar hydrophobic synthetic agonist for estrogen receptors. The chemical properties of EE2 are outlined in Table 2. EE2 is the primary estrogen used in combined oral contraceptives and hormone replacement therapy. In humans, EE2 has been reported to have one to two times greater affinity for nuclear estrogen receptors and up to five times greater affinity for nuclear estrogen receptors in some fish species than E2 (Aris *et al.*, 2014).

Frequent use has made EE2 a common contaminant in surface waters. It primarily enters through WWTPs, septic systems, and agricultural runoff (Aris *et al.*, 2014). Focusing on WWTPs, EE2 is excreted as free EE2 or as a glucuronide-EE2 conjugate after human consumption, and enters WWTPs. Due to its stability, minimal degradation of EE2 occurs in the WWTP, however, bacterial de-conjugation of EE2-glucuronides may occur (Andersen *et al.*, 2003; Parrott and Blunt, 2005; Aris *et al.*, 2014). Contaminated effluent is then released into surface waters with resulting EE2 concentrations in the low ng/L range (Kolpin *et al.*, 2002; Aris *et al.*, 2014). In recent years, upgrades to the Kitchener WWTP, have helped reduce effluent estrogen equivalents (Hicks *et al.*, 2017). However, due to the

high biological activity and presence in the aquatic environment, the toxicity of EE2 to non-target organisms has been and will continue to be extensively studied (Aris *et al.*, 2014; Overturf *et al.*, 2015).

Table 2: Chemical properties of 17β-Estradiol (E2) and 17α-Ethinylestradiol (EE2)

	17β-Estradiol (E2)	17α-Ethinylestradiol (EE2)
Chemical Structure		
Molecular Formula	$C_{18}H_{24}O_2$	$C_{20}H_{24}O_2$
Molecular Weight (g/mol)	272.382	296.403
Aqueous Solubility (mg/L at 27° C)	3.6	11.3
Log K_{OW}	4.01	3.67
pKa	0.88 and 10.33	-1.7 and 10.33

- Data obtained from https://www.drugbank.ca/; pKa values are predicted.

1.6.1.1. EE2 Mode of Action

In humans, the proposed mode of action of oral contraceptives is through the activation of nuclear estrogen receptors in the pituitary and hypothalamus. This initiates a negative feedback loop along the hypothalamus-pituitary-gonadal (HPG) axis, thereby inhibiting the production of gonadotropin releasing hormone (GnRH). A reduction of GnRH limits the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH), GtH1 and GtH2 in fish, from the pituitary gland, (Rivera *et al.,* 1999; Mateos *et al.*, 2002). This reduces the production of endogenous estrogen 17β-estradiol (E2) and inhibits mid-cycle peaks (Rivera *et al.,* 1999; Mateos *et al.*, 2002). This results in the inhibition of oocyte maturation and ovulation, thereby reducing the likelihood of conception in women (Rivera *et al.*, 1999).

A novel EE2 mode of action is though the activation of G protein-coupled estrogen receptor (GPER). Little is known about the impact of GPER activation by EE2 in mammalian models. To the best of our knowledge, no studies have examined the impact of GPER activation by EE2 in fish. Understanding another mechanism of action for EE2 is important for understanding the risk posed to non-target organisms. The role and regulation of E2 and GPER is outlined in greater detail in Section 1.4.4.

1.6.1.2. Toxicity of EE2 to Fish

In fish, EE2 is a potent toxicant, significantly influencing both fecundity and secondary sexual characteristics. Effects on fecundity appear to be bi-modal and dependent on concentration (reviewed by Overturf *et al.,* 2015). In a 21 day exposure of mature fathead minnows (*Pimephales promelas*), EE2 increased egg production at 0.1 ng/L and 1

ng/L, however a significant decrease was observed at 100 ng/L in the same study (Pawlowski *et al.*, 2004). This is supported by other studies with varying exposure lengths and test species. In fathead minnows exposed for their full life-cycle, an increase in egg production was observed at 0.32 ng/L and 0.96 ng/L, which was contrasted with a cessation of egg production when exposed to concentrations greater than 3.5 ng/L (Parrott and Blunt, 2005). A study that exposed adult Japanese medaka for 14 days to EE2, also observed an increase in fecundity at 0.2 ng/L EE2 and a reduction at 500 ng/L EE2 (Tilton *et al.*, 2005).

In each of the three studies listed above, a reduction in fertilization was observed upon EE2 exposure, indicating potential male reproductive impairment. This was supported by the feminization of male sexual and secondary sexual characteristics observed in studies by Parrot & Blunt (2005) and Pawlowski *et al.*, (2004), and a whole lake population collapse as reported by Kidd *et al.*, (2007).

In an attempt to characterize the environmental relevance of EE2 toxicity in wild fish, Kidd *et al.*, 2007, conducted a 7 year whole lake study in the Experimental Lakes Area in Northwestern Ontario, Canada. They exposed the lake to 5-6 ng/L EE2 and observed an induction in VTG in both male and female fathead minnows, and the development of intersex gonads in male testicular tissue (Kidd *et al.*, 2007). This exposure led to the near extinction of fathead minnows from the lake. Interestingly however, by the spring of the fourth year post EE2 treatment, the number of fathead minnows returned back to baseline and testicular abnormalities were not present (Blanchfield *et al.*, 2015).

EE2 toxicity to fish is extensive and shows significant impacts to apical endpoints. Effects have been primarily focused on nuclear estrogen receptor activation along the HPGL axis in fish. Many studies attempt to explain observed toxicity to changes in genetic

expression or protein abundance. To date no studies have investigated the toxicity of EE2 to membrane estrogen receptors in fish, specifically GPER. This may be a novel mode of action for EE2 since environmental estrogens have been shown to activate GPER (Thomas and Dong, 2006).

As described above, GPER activation is a non-genomic mode of action that may result in different avenues of toxicity. An example of a GPER mediated endpoint is embryonic heart rate. Currently only one study has examined the impact of EE2 on fish heart rate. This study observed a significant decrease in embryonic heart rate of zebrafish exposed to 0.4 ng/L EE2 (Santos *et al.*, 2014). These results are supportive of the finding that EE2 results in reduced blood circulation in embryonic medaka (Schiller *et al.*, 2014). However, it contradicts the observed impact of E2 which at 1 mg/L resulted in an increase in embryonic zebrafish heart rate (Romano *et al.*, 2017). Although they are different compounds, structural similarity and the ability of both chemicals to activate GPER suggests that their toxicity may be related (Table 2). A potential explanation for this discrepancy could be the bi-modal effects that are often observed with estrogen receptor activation. In these experiments, the E2 exposure was approximately 1 million times greater than the EE2 exposure. It is important to note that concentrations in the low ng/L range are more representative of what is observed in surface waters. One research objective of this thesis is to better understand the toxicity of EE2 to the embryonic heart rate of fish at environmentally relevant concentrations.

1.6.2. Cyclodextrins

Cyclodextrins are a class of α-(1,4) cyclically linked glucopyranose oligosaccharides. Cyclodextrins are most commonly present as either α, β, or γ containing 6, 7, or 8 glucopyranose subunits, respectively (Table 3) (Del Valle, 2004). The resulting toroidal configuration of the cyclodextrin molecule has been proven to be a very useful binding cavity (Szejtli, 1998; Davis and Brewster, 2004). Binding within the central cavity of the cyclodextrin molecule is known as "guest inclusion" where the guest molecule is non-covalently bound to the cyclodextrin cavity and is reversible. Contributing to the utility of cyclodextrins is that they are amphiphilic since the outer portion of the toroid is more hydrophilic than the cavity (Szejtli, 1998). This encourages passive non-covalent inclusion of whole lipophilic molecules or lipophilic moieties of those molecules in the cavity of cyclodextrins in an aqueous solution (Szejtli, 1998; Loftsson and Brewster, 2012). Inclusion within the cyclodextrin compound has been shown to dramatically improve the aqueous solubility of poorly soluble compounds (Loftsson and Brewster, 2012). Cyclodextrins are currently used in odour control sprays, solubilizers, and excipients in the pharmaceutical industry (Loftsson and Brewster, 2012). As of 2012, cyclodextrins are have been used in 35 different pharmaceuticals (Loftsson and Brewster, 2012).

1.6.2.1. Hydroxypropyl-β-Cyclodextrin (HPβCD)

Of the three conformations of cyclodextrins, α, γ, and β, the β conformation has the most useful cavity size. However, β-cyclodextrin is the least water soluble, which limits its ability to solubilize guests (Loftsson and Brewster, 2012). As a result, modifications have been made to β-cyclodextrin by adding hydroxypropyl side chains, creating

hydroxypropyl-β-cyclodextrin (HPβCD). This addition increased the polarity of the outer region of the toroid thus improving the aqueous solubility (Table 3) (Szejtli, 1998; Loftsson and Brewster, 2012). HPβCD is currently used as an excipient in the pharmaceutical industry, and as the active ingredient in the deodourizer Febreze®. HPβCD can be found in oral, parenteral, rectal, and ophthalmic drugs, primarily increasing the solubility and bioavailability of the active pharmaceutical (Loftsson and Brewster, 2012). Although the theoretical inclusion of guests occurs at a 1 : 1 molar ratio with HPβCD, a review of inclusion efficiencies mentions that the optimal molar ratio for most guests is approximately 4 : 1, HPβCD : Guest (Loftsson and Brewster, 2012). With minimal clinical side effects and a low oral bioavailability of 3 %, HPβCD has generally been deemed safe for human consumption (Loftsson and Brewster, 2012).

Table 3: Chemical properties, and cavity dimensions of α, β, and γ – cyclodextrin. Modified from (Szejtli, 1998; Loftsson and Brewster, 2012).

Property	α	β	γ	HPβCD
Number of Glucopyranose Units	6	7	8	7
Molecular Weight (g/mol)	972	1135	1297	1396
Aqueous Solubility *	14.5	1.85	23.2	45
Cavity Diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3	6.0-6.5
Height of Torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Approximate Cavity Volume ($Å^3$)	174	262	427	262

* Solubility represented as g per 100 ml of water at room temperature.

1.6.2.2. HPβCD Toxicity to Fish

Environmental concern has risen with the increase in the use of HPβCD in pharmaceuticals and as deodourizers in laundry detergents. However, to the best of our knowledge, neither effluent of surface water concentrations are known. To date, there has only been one study that has examined the environmental impacts of HPβCD to fish. This study exposed American flagfish (*Jordanella floridae*) to HPβCD for their complete life-cycle (Anderson *et al.*, 2016). It was observed that there was no impact to fecundity, however, an increase in female gonadosomatic index (GSI) was observed at 5 µg/L. Furthermore, a decrease in offspring growth and tolerance to copper was observed after parental exposure to HPβCD (Anderson *et al.*, 2016). Currently, the mechanism behind this increase in female GSI is not understood. However, there is potential that HPβCD is complexing with endogenous steroid hormones and or pheromones.

1.6.2.3. HPβCD and EE2

HPβCD forms complexes with estrogens in aqueous solutions (Pérez and Escandar, 2013). EE2 has a greater stability and a higher association constant with HPβCD relative to endogenous estrogens (Pérez and Escandar, 2013). This can be attributed to the greater hydrophobicity of EE2 (Table 2). Stability typically improves with increased hydrophobicity of the guest molecule (Pérez and Escandar, 2013).

Given the low water solubility of EE2, organic solvents are often used as a carrier when assessing their toxicity in an aquatic setting. However, complexation between EE2 and HPβCD is reduced in the presence of organic solvents. Organic solvents have the

ability to interact with the binding cavity of cyclodextrins, disrupting stability. These effects are most prominent at organic solvent concentrations greater than 20 % V/V (Pérez and Escandar, 2013). It is recommended that for aquatic toxicity studies organic solvents used as carriers should be no greater than 0.01 % V/V. The influence of organic solvents to the inclusion of EE2 into HPβCD in these studies is expected to be minimal, however, the possibility should still be taken into consideration.

Recent interest in the ability of HPβCD to influence the toxicity of guest compounds has increased. It has been shown that HPβCD can suppress the estrogenic effects of E2 in a yeast two-hybrid assay (Oishi *et al.*, 2008). HPβCD had no impact on E2 estrogen receptor affinity in this study, suggesting that HPβCD may be blocking E2 from crossing biological membranes (Oishi *et al.*, 2008).

1.7. General Materials and Methods

1.7.1. Animal Husbandry

Below is a brief description of the animal husbandry that took place during this study. The breeding stock of medaka were held and maintained under the approved animal usage protocol number AUP 14-002, by the Animal Care Committee at the University Of Ontario Institute Of Technology, *Oshawa, Ontario, Canada*. All animal care was conducted in accordance with the Canadian Council on Animal Care (CCAC).

1.7.1.1. Lab Water Process and Lighting

Unless otherwise stated, the 25° C laboratory water used to house fish for this project consisted of municipal water (*Oshawa, Ontario*), de-chlorinated via charcoal and resins, and buffered with calcium carbonate. After treatment, the water is pumped to a header tank above the laboratory, heated to 25° C, and then gravity fed down to the aquaria. A ball valve on the incoming water line regulates the flow into the aquaria, and stand pipes allow for water to exit into a trough. The flows are typically set to 5 turnovers per day, resulting in a > 99 % molecular turnover per day (Sprague, 1969).

The laboratory experienced a 16 hour light and 8 hour dark photoperiod including a half hour dawn and dusk period included in the light phase. Lighting consisted of full spectrum fluorescent tubes. Lighting was maintained this way for the complete duration of the study.

1.7.1.2. Japanese medaka

Japanese medaka (FLFII) were originally obtained from the National Institute for basic Biology (*Okazaki, Japan*). The general lab population of medaka were housed in 25 ± 1° C, 70 L flow though aquaria at a density of approximately 0.5 fish per L. They were fed Tetramin^{TM} *Pro Flake* (Tetra United Pet Group), (minimum crude protein of 46.0 %, 12.0 % crude fat, 3.0 % crude fiber, 1.1 % phosphorus, 200 mg/kg ascorbic acid, and maximum moisture of 8.0 %) flake food and freshly hatched brine shrimp nauplii twice per day. Premium brine shrimp eggs were purchased from Brine Shrimp Direct (*Ogden, Utah, USA*).

Once fish reach sexual maturity they were separated into breeding harems consisting of 15 males and 15 females per 70 L aquarium. Egg collection occurred once per day, any females carrying eggs were netted and the clutch of eggs gently removed before returning the fish to the aquarium. Additionally, all of the greenery in the tanks was removed, inspected for deposited egg clutches, rinsed and returned to the aquarium. Water from the breeding aquaria was then siphoned into a shallow bucket and any loose eggs kept. The eggs were then rolled on a wetted piece of paper towel to reduce the adherence of the attaching filaments that cover the eggs. The eggs were then placed in 150 mL crystallization dishes containing rearing solution comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. The crystallization dishes were placed in a 27° C temperature control room on an Advanced Digital Skater (VWR International, *Mississauga, Ontario, Canada*) set to 60 RPM to ensure uniformity of hatching time. After 24 h of incubation, any unfertilized eggs characterized by a blue colouration were removed. The remaining embryos were either left

to develop until hatch 9 – 11 days post fertilization and used to restock the lab population, or used in an embryonic toxicity test. Embryonic toxicity test protocol is outlined in the following sections. For the laboratory population, hatched fry were placed in a 20 L flow through aquaria and fed as described above. Once they reached an average length of approximately 1 cm, they were transferred into 70 L aquaria at a density of 0.5 fish per L until sexual maturity.

1.8. General Aims, Rationale, and Knowledge Gaps

The overall goals of this research thesis were to better understand the non-classical toxicity pathways of estrogens in teleost fish, and the influence of the deodourizer (HPβCD) on EE2 toxicity. This project was broken down into three major aims, each of which are described in brief below, and in greater detail in chapters 2 – 4.

The first major aim was to determine if EE2 could alter the embryonic heart rate of Japanese medaka. There has been evidence that E2 can influence embryonic heart rate through GPER activation in embryonic zebrafish. EE2 can act as a ligand for GPER, suggesting that GPER may be a novel mechanism of action of EE2 in fish. To the best of our knowledge, only one study has reported a decrease in heart rate following EE2 exposure to zebrafish embryos at environmentally relevant concentrations. We also wanted to determine if specific GPER agonism or antagonism can modulate embryonic heart rate in Japanese medaka.

The second major aim was to determine if HPβCD could alter the above observed toxicity to embryonic medaka. HPβCD has been shown to include EE2 as a guest, however, alterations in guest toxicity to non-target organisms has yet to be understood. To the best

of our knowledge, there has only been one study examining the toxicity of HPβCD alone to fish (Anderson *et al.*, 2016). This research will help elucidate the influence of HPβCD on the toxicity of other known toxicants, and is important to the field of aquatic toxicology as it will help further the understanding of the combinatorial toxicity of PPCPs.

Finally, the last aim of this project was to determine if the observed alterations in embryonic heart rate caused by the exposure to EE2 result in metabolic changes within the organism. Heart rate is commonly used as a surrogate measurement for metabolic rate. Through the use of a microplate respirometer, we aimed to determine if the observed changes in heart rate following EE2 exposure corresponded to changes in oxygen consumption (MO_2).

Chapter 2: EE2 and HPβCD Impact on Embryonic Heart Rate

2. Chapter 2: EE2 and HPβCD Impact on Embryonic Heart Rate

2.1. Introduction

The consumption of pharmaceutical and personal care products continually increases annually. Over the last two decades, research into the toxicological effects of these compounds in non-target organisms has been extensive. Very few pharmaceutical compounds have been as well studied from an environmental perspective as 17α-ethinylestradiol (EE2) (Andersen *et al.*, 2003; Parrott and Blunt, 2005; Kidd *et al.*, 2007; Aris *et al.*, 2014; Overturf *et al.*, 2015). It has been repeatedly demonstrated that EE2 interferes with fertility and fecundity in many fish species through interference in the hypothalamus pituitary gonadal axis (Rivera *et al.*, 1999; Mateos *et al.*, 2002). Activation of nuclear membrane estrogen receptors ERα and ERβ is known as classical estrogen signaling (Prossnitz and Barton, 2014). This mode of action typically results in the alteration of estrogen related genes, often leading to the presence of female secondary sexual characteristics in genotypic males. These effects are often slower to appear due to the inherent time it takes for gene up or down regulation, transcription, and translation into respective proteins (Prossnitz and Barton, 2014). The effects of EE2 toxicity through classical estrogen signaling can be significant at very low concentrations, and they are often the result of chronic exposure (Kidd *et al.*, 2007; Overturf *et al.*, 2015).

Recently, the characterization of the G protein coupled estrogen receptor (GPER) has provided an alternative mode of action for E2 (Romano *et al.*, 2017). GPER is a membrane bound estrogen receptor that is located on the sarcoplasmic reticulum (Prossnitz and Barton, 2014; Diamante *et al.*, 2017). Due to the nature of G Protein coupled receptors,

the cellular response is often more immediate (Gaudet *et al.*, 2015; Diamante *et al.*, 2017; Sharma *et al.*, 2018). GPER has been localized to many tissues within vertebrate models, and one of the more prominent localizations of GPER is in the cardiovascular system (Sharma *et al.*, 2018). Activation of GPER has been shown to alter heart rate as well as blood flow in embryonic zebrafish (Santos *et al.*, 2014; Romano *et al.*, 2017). The heart rate of embryonic zebrafish exposed to 1 mg/L E2 increased while 0.4 ng/L EE2 resulted in a decrease (Santos *et al.*, 2014; Romano *et al.*, 2017). It was further demonstrated that the increase in heart rate was the result of GPER activation and not classical estrogen signaling at high concentrations of E2 (Romano *et al.*, 2017).

The primary goal of this research was to understand the impact of environmentally relevant concentrations of EE2 on the heart rate of embryonic Japanese medaka. Previous research had indicated that GPER activation can increase heart rate at high concentrations, however the mechanism of action for low levels of EE2 causing decreased heart rate had not been investigated. The effects of such low level toxicity through a novel mode of action is very valuable for understanding the full sub-lethal effects of EE2 within the environment.

In addition to investigating the influence of GPER on embryonic heart rates, the ability of hydroxypropyl-β-cyclodextrin (HPβCD) to alter the observed toxicity of EE2 was also examined. HPβCD is an odour suppressant and an excipient commonly used in detergents and in drug delivery systems (Szejtli, 1998; Del Valle, 2004; Loftsson and Brewster, 2012). It exhibits a toroidal structure with amphiphilic properties, the inner region being hydrophobic while the outer region is hydrophilic (Szejtli, 1998). The structure and variance in hydrophobicity creates a cavity that other hydrophobic compounds may bind to, aiding in solubility (Szejtli, 1998). Cyclodextrins are thought to

be non-toxic and are often used to aid in the distribution of drugs throughout the body through inclusion (Del Valle, 2004). They are also popular in odour suppressants as they can encapsulate molecules, preventing them from interacting with olfactory receptors (Del Valle, 2004). It is hypothesized HPβCD can include EE2 potentially preventing it from entering the embryo or interacting with receptors (Oishi *et al.*, 2008; Pérez and Escandar, 2013). Alternatively, HPβCD may improve the solubility of EE2 and bioavailability to the embryo.

2.2. Objectives, Knowledge Gaps, Rationale, and Hypotheses

2.2.1. Objective 1: Embryonic Heart Rate: EE2 and HPβCD – Pilot Studies

The goal of objective 1 was to determine if EE2 exposure can alter the heart rate of embryonic Japanese medaka. Evidence suggests that EE2 can activate GPER and GPER can regulate embryonic heart rate in zebrafish. We aimed to determine an effect concentration for this endpoint. Additionally, we wanted to determine if HPβCD has the ability to alter any EE2 induced changes in embryonic heart rate.

The null hypotheses for this experiment were:

H_0: There is no difference between the heart rates of embryonic Japanese medaka exposed to EE2 versus the carrier control.

H_0: There is no difference between the heart rates of embryonic Japanese medaka exposed to EE2 versus EE2 + HPβCD.

2.2.2. Objective 2: Sex Comparison

The goal of objective 2 was to compare male and female embryonic heart rates following exposure to EE2. The FLFII strain of Japanese medaka presents the unique ability for researchers to determine sex as early as 48 hpf. Given that EE2 is a potent estrogen, there exists the potential that EE2 exposure may result in differential impacts to the heart rates of embryonic male and female medaka. This research was aimed at reducing variability in the data and identifying sex specific effects of EE2 on embryonic heart rates.

The null hypotheses for this experiment were:

H_0: There is no difference between the heart rates of male and female embryonic Japanese medaka following an exposure to EE2.

H_0: There is no difference between the heart rates of embryonic Japanese medaka exposed to EE2 versus the carrier control.

2.2.3. Objective 3: ERα, ERβ, and GPER Agonist and Antagonist Impact on Embryonic Heart Rate.

Objective 3 was completed to establish the mode of action of EE2 on embryonic heart rates. Data suggests that GPER activation is responsible for the alteration in heart rates of embryonic zebrafish (Romano *et al.*, 2017). This study aimed at confirming these results with embryonic Japanese medaka exposed to agonists and antagonist of ERα, ERβ, and GPER alone and in combination with EE2.

The null hypothesis for this study was:

H_0: The agonist, antagonists, or EE2 have no impact on the heart rate of embryonic Japanese medaka.

2.2.4. Objective 4: The Influence of HPβCD on EE2 Toxicity

The aim of objective 4 was to determine the optimal ratio of EE2:HPβCD. By exposing medaka to a range of EE2:HPβCD ratios, the identification of a concentration response may help support the theory that HPβCD can alter the toxicity of the included guest.

The null hypothesis for this experiment were:

H_0: There is no difference between the heart rate of embryonic Japanese medaka exposed to various ratios of EE2 and HPβCD.

2.3. Materials and Methods

2.3.1. Objective 1: Embryonic Heart Rate: EE2 and HPβCD – Pilot Studies

2.3.1.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), was dissolved in acetone. EE2 alone treatments were made through serial dilutions with rearing solution as the diluent. For the first pilot study, an EE2 alone rangefinder, final concentrations of 5, 50, 500, 5000, and 50000 ng/L of EE2 were prepared. For the EE2 + HPβCD pilot study, final nominal concentrations of 0 ng/L (carrier control), 0.1 ng/L, 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 were made. HPβCD, 97.0 %, was purchased from Acros Organics (*Thermo-Fisher Scientific*), and dissolved in deionized water. HPβCD alone treatments were also made using serial dilutions with rearing solution as a diluent with final nominal concentrations set at 4x molar concentration of EE2 alone, corresponding to 0 ng/L (carrier control), 1.7 ng/L, 17 ng/L, 170 ng/L, 1700 ng/L and 17000 ng/L HPβCD. EE2 + HPβCD treatments were made using the same methodology listed above with final nominal concentrations of combination 0 + 0 ng/L (carrier control), 0.1 + 1.7 ng/L, 1 + 17 ng/L, 10 + 170 ng/L, 100 + 1700, and 1000 + 17000 ng/L EE2 + HPβCD. A summary of exposure concentrations can be found in Table 4. A carrier control of 0.01 % v/v acetone was used, and this concentration of acetone was kept constant across all treatments. Solutions were made 24 hours prior to initial exposure and stored in amber glass bottles and renewed once every 24 hours.

Table 4: Range finding study nominal treatment concentrations of 17α-ethinylestradiol (EE2) alone, hydroxypropyl-β-cyclodextrin (HPβCD) alone, and a mixture of EE2 + HPβCD exposed to embryonic Japanese medaka (*O. latipes*).

[EE2] Alone (ng/L)	[HPβCD] Alone (ng/L)	[EE2] + [HPβCD] (ng/L)	Acetone (%)	Embryos Per Well	Number of Wells
0	0	0 + 0	0.01	15	1
0.1	1.7	0.1 + 1.7	0.01	15	1
1	17	1 + 17	0.01	15	1
10	170	10 + 170	0.01	15	1
100	1700	100 + 1700	0.01	15	1
1000	17000	1000 + 17000	0.01	15	1

2.3.1.2. Organisms and Experimental Procedure

Adult Japanese medaka (FLFII), originally obtained from the National Institute for basic Biology (*Okazaki, Japan*) were bred in house as described in section 1.7.1.2. Collected embryos were reared in rearing solution, section 1.7.1.2, inside a 27˚ C temperature control room with a 16h:8h light:dark photoperiod and 0.5 h of dawn and dusk included in the light phase.

For the EE2 alone pilot study, 6 hpf embryos were exposed to 0, 5, 50, 500, 5000, and 50000 ng/L EE2 in 6 well plates. There were 3 replicates per treatment with 9 embryos per replicate. The heart rate of each embryo was measured at 120hpf. Heart rate was determined by placing all embryos from a randomly selected replicate on a petri dish, and allowing to acclimate for 2 minutes before viewing under a Leica stereo microscope (Leica, *Concord, Ontario, Canada*). The number of heart beats that occurred in 15 seconds for each embryo was determined and then converted to beats per minute by multiplying by four. To normalize the data, the percent difference from the respective carrier control was calculated and used for analysis (Equation 1).

There were three treatment groups for the EE2 and HPβCD range finding study: EE2 alone, HPβCD alone, and EE2 and HPβCD. At 6 hpf, the embryos were separated and exposed to the various treatment groups (Table 4) in a single six well plate with 15 embryos per treatment. Each well contained 10 ml of exposure solution and was changed once every 24 hours. Heart rate was monitored one every 24 hours from 72 – 196 hpf.

Data were analyzed by calculating the mean heart rate in beats per minute for each treatment and tested for normality and homogeneity. A two-way ANOVA with a Dunnett's

post hoc test was used to determine differences from the respective carrier control as well as any differences between HPβCD, EE2, and EE2 + HPβCD treatments. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) and run at an alpha of 0.05.

$$\%\ Difference\ of\ Carrier\ \ Control = \left(\left(\frac{Treatment\ Heart\ Rate}{Mean\ Heart\ Rate\ of\ Carrier\ Control}\right) * 100\right) - 100$$

Equation 1: Calculation of the % difference in heart rate from carrier control. Where treatment heart rate is the heart rate from an individual embryo in beats per minute, and the mean heart rate of carrier control is the average heart rate of the carrier control in beats per minute.

2.3.2. Objective 2: Sex Comparison

2.3.2.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), was dissolved in acetone. EE2 treatments were made through serial dilutions with rearing solution as the diluent, and final nominal concentrations of 0 ng/L (carrier control), 0.1 ng/L, 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 were made. Acetone was kept constant in all treatments at a concentration of 0.01 % v/v. Solutions were made 24 hours prior to exposure and stored in amber glass bottles in a 27° C temperature control room for the duration of the experiment. A summary of exposure concentrations can be found in Table 5.

2.3.2.2. Experimental Procedure

Embryonic Japanese medaka were obtained and maintained as described in section 1.7.1.2. At 24 hpf, 20 embryos per treatment were exposed in a 6 well plate to a range of EE2 concentrations, 10 ml per well. At 72 hpf, the sex of each embryo was determined using a Leica DM2000 Fluorescence Microscope (Leica, *Concord, Ontario, Canada*), and separated into male and female exposures. Embryos were thinned to 7 embryos per sex per treatment per replicate (Table 5).

Embryonic heart rate was monitored once every 24 hours beginning at 120 hpf and ending at hatch (216 hpf). Heart rate was determined by placing all 7 embryos from a

randomly selected single treatment on a petri dish. The embryos were acclimated for 2 minutes before viewing under viewed under a Leica stereo microscope (Leica, *Concord, Ontario, Canada*). The number of heart beats that occurred in 20 seconds was determined and then converted to beats per minute by multiplying by three. The percent difference from the carrier control was calculated and used for analysis (Equation 1). This experimental procedure was replicated independently three times.

Data were analyzed by calculating the mean heart rate in beats per minute for each treatment group and compared using an ANOVA. If the data was not normally distributed, a Kruskal-Wallis one way ANOVA on ranks test was completed. An all pairwise multiple comparison Tukey *post hoc* test was used to determine differences between treatments. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) at an alpha of 0.05.

Table 5: Exposure summary for Objective 2: sex comparison. Nominal concentrations of 17α-ethinylestradiol (EE2) exposed to male and female embryonic Japanese medaka (*O. latipes*), number of replicates, and number of embryos per replicate per sex.

Treatment #	[EE2] (ng/L)	Acetone (%)	Number of replicates	Number of embryos per replicate per sex
1	0	0.01	3	7
2	0.1	0.01	3	7
3	1	0.01	3	7
4	10	0.01	3	7
5	100	0.01	3	7
6	1000	0.01	3	7

2.3.3. Objective 3: ERα, ERβ, and GPER Agonists and Antagonists Impact on Embryonic Heart Rate

2.3.3.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), G-1 (rel-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone) from Cayman Chemical (*Ann Arbor, Michigan, USA*), Fulvestrant (ICI) ((7α,17β)-7-[9-[4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) from Toronto Research Chemicals inc. (*Toronto, Ontario, Canada*), G-36 ((4S)-rel-4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4,5,9,bS-tetrahydro-8-(1-methylethyl)-3H-cyclopenta[c]quinolone) from Cayman Chemical (*Ann Arbor, Michigan, USA*), PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol-2-phenyl-3-4hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine) from Sigma-Aldrich (*Oakville, Ontario, Canada*), and ZK 154015 (2-(4-Hydroxyphenyl)-3-methyl-1-[10-(pentylsulfonyl)decyl]-1H-indol-5-ol) from Tocris (*Oakville, Ontario, Canada*), were dissolved in acetone. Treatments of each chemical (EE2, G-1, Fulvestrant (ICI), G-36, PHTPP, and Zk 164015), were made through serial dilutions with rearing solution as the diluent. Final nominal concentrations for each compound were, 10 ng/L, and 100 ng/L with 0.01 % v/v acetone being kept constant in all treatments. Solutions were made 24 hours

prior to exposure and stored in amber glass bottles in a 27° C temperature control room for the duration of the experiment.

2.3.3.2. Experimental Procedure

Below is the general procedure for each agonist/antagonist, this procedure was conducted for G-1, Fulvestrant (ICI), G-36, PHTPP, and ZK 16405.

Embryonic Japanese medaka were obtained and maintained as described in section 1.7.1.2. At 24 hpf, 7 embryos per treatment were exposed in a 6 well plate to a carrier control (0.01 % v/v acetone), 10 ng/L EE2, 10 ng/L agonist or antagonist, 100 ng/L agonist/antagonist, 10 ng/L EE2 + 10 ng/L agonist/antagonist, 10 ng/L EE2 + 100 ng/L agonist/antagonist, with 10 ml of treatment solution per well. At 48 hpf, embryos were thinned to 7 embryos per replicate with three replicates per treatment (Table 6).

Embryonic heart rate was monitored once every 24 hours beginning at 120 hpf and ending at hatch (192–216 hpf). Heart rate was determined by placing all 7 embryos from a randomly selected single treatment on a petri dish. The embryos were acclimated for 2 minutes before viewing under viewed under a Leica stereo microscope (Leica, *Concord, Ontario, Canada*). The number of heart beats that occurred in 20 seconds was determined and then converted to beats per minute by multiplying by three. The percent difference from the carrier control was calculated and used for analysis (Equation 1).

Data were analyzed by calculating the mean heart rate in beats per minute for each treatment group and compared using an ANOVA for Fulvestrant, G-36, and PHTPP experiments. Data was not normally distributed in the G-1 and ZK 16405 experiments, and a Kruskal-Wallis one way ANOVA on ranks test was completed. An all pairwise multiple

comparison Tukey *post hoc* test was used to determine differences between treatments. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) at an alpha of 0.05.

Table 6: Exposure summary for Objective 3: ERα, ERβ, and GPER agonists and antagonists. Nominal concentrations of 17α-ethinylestradiol (EE2), and agonist/antagonist (G-1, Fulvestrant (ICI), G-36, PHTPP, or ZK 164015) exposed to embryonic Japanese medaka (*O. latipes*), number of replicates, and number of embryos per replicate.

Treatment #	[EE2] (ng/L)	[Agonist/Antagonist] (ng/L)	Acetone (%)	Number of replicates	Number of embryos per replicate
1	0	0	0.01	3	7
2	10	0	0.01	3	7
3	0	10	0.01	3	7
4	0	100	0.01	3	7
5	10	10	0.01	3	7
6	10	100	0.01	3	7

2.3.4. Objective 4: The Influence of HPβCD on EE2 Toxicity

2.3.4.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), was dissolved in acetone, and Hydroxypropyl-β-cyclodextrin (HPβCD), 97.0 %, was purchased from Acros Oganics (*New Jersey, USA*) was dissolved in deionized water. Treatment solution were made using rearing solution as a diluent. Nominal treatments consisted of a carrier control, 10 ng/L EE2, 188 ng/L HPβCD, 10 ng/L EE2 + 47 ng/L HPβCD, 10 ng/L EE2 + 94 ng/L HPβCD, and 10 ng/L EE2 + 188 ng/L HPβCD. An acetone carrier concentration of 0.01 % v/v was maintained in each treatment.

2.3.4.2. Experimental Procedure

Embryonic Japanese medaka were obtained and maintained as described in section 1.7.1.2. At 24 hpf, 12 embryos per treatment were exposed in a 6 well plate to a carrier control (0.01 % v/v acetone), 10 ng/L EE2, 188 ng/L HPβCD, 10 ng/L EE2 + 47 ng/L HPβCD, 10 ng/L EE2 + 94 ng/L HPβCD, and 10 ng/L EE2 + 188 ng/L HPβCD, with 10 ml of treatment solution per well. At 48 hpf, embryos were thinned to 7 embryos per treatment (Table 7).

Embryonic heart rate was monitored once every 24 hours beginning at 120 hpf and ending at hatch (192–216 hpf). Heart rate was determined by placing all 7 embryos from a

randomly selected single treatment on a petri dish. The embryos were acclimated for 2 minutes before viewing under a Leica stereo microscope (Leica, *Concord, Ontario, Canada*). The number of heart beats that occurred in 20 seconds was determined and then converted to beats per minute by multiplying by three. The percent difference from the carrier control was calculated and used for analysis (Equation 1).

Data were analyzed by calculating the mean heart rate in beats per minute for each treatment group and compared using an ANOVA. The data was not normally distributed, and a Kruskal-Wallis one way ANOVA on ranks test was completed. An all pairwise multiple comparison Tukey *post hoc* test was used to determine differences between treatments. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) at an alpha of 0.05.

Table 7: Exposure summary for Objective 4: The Influence of HPβCD on EE2 Toxicity. Nominal concentrations of 17α-ethinylestradiol (EE2), and hydroxypropyl-β-cyclodextrin (HPβCD) exposed to embryonic Japanese medaka (*O. latipes*), number of replicates, and number of embryos per replicate.

Treatment #	[EE2] (ng/L)	[HPβCD] (ng/L)	Acetone (%)	Number of replicates	Number of embryos per replicate
1	0	0	0.01	3	7
2	10	0	0.01	3	7
3	0	188	0.01	3	7
4	10	47	0.01	3	7
5	10	94	0.01	3	7
6	10	188	0.01	3	7

2.4. Results

2.4.1. Embryonic Heart Rate: EE2 and HPβCD – Pilot Studies

Embryonic Japanese medaka were exposed to a range of EE2 concentrations in the initial pilot study. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. Mean heart rate, represented as percent change from the control, was assessed at 120 hpf. Control embryos had a mean heart rate of 100.0 beats per minute. Embryos exposed to 50, 500, and 5000 ng/L EE2 experienced a significant decrease in heart rate, 6.0, 6.6 and 5.4 % respectively, compared to the control ($P \leq 0.05$) (Figure 2), while embryos exposed to 50000 ng/L EE2 experienced a 16.7 % increase in heart rate, which was significantly greater than the control ($P \leq 0.05$) (Figure 2).

For the range finding study, embryonic medaka were exposed to various concentrations of EE2, HPβCD, or a combination of EE2 and HPβCD. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. Heart rate was monitored every 24 h from 72 to 196 hpf, (Figure 3). At 72 hpf embryos exposed to EE2 alone experienced a significant decrease in heart rate compared to the carrier control at 10 ng/L, 100 ng/L, and 1000 ng/L EE2 in a concentration dependent manner ($P \leq 0.05$) (Figure 3A). The largest decrease in heart rate, -11.2 %, occurred at 1000 ng/L EE2. No significant differences were observed in embryos exposed to HPβCD compared to the carrier control alone at 72 hpf ($P > 0.05$) (Appendix: Figure S19). All combinations of EE2 and HPβCD treatments resulted in decreased heart rates in a concentration dependent manner 0.1 + 1.7 ng/L, 1 + 17 ng/L, 10 + 170, 100 + 1700 ng/L, and 1000 + 17000 ng/L (EE2 + HPβCD) ($P \leq 0.05$) (Figure 3A). Embryos exposed to EE2 + HPβCD at 0.1 + 1.7 ng/L and 1 + 17 ng/L

treatments had significantly lower heart rates than embryos exposed to EE2 alone at 0.1 ng/L and 1 ng/L ($P \leq 0.05$) Figure 3A.

A similar trend of decreasing heart rate was observed at 96 hpf. A reduced heart rate was observed in embryos exposed to 100 ng/L and 1000 ng/L EE2 alone compared to the carrier control ($P \leq 0.05$) (Figure 3B). Embryos exposed to HPβCD experienced no significant difference compared to the carrier control ($P > 0.05$) (Figure S19). A reduced heart rate occurred in embryos exposed to 1 + 17 ng/L, 10 + 170 ng/L, 100 + 1700 ng/L, and 1000 + 17000 ng/L of EE2 + HPβCD compared to the carrier control ($P \leq 0.05$) (Figure 3B). Embryos exposed to EE2 + HPβCD at 1 + 1.7 ng/L had significantly lower heart rates than in embryos exposed to 0.1 ng/L EE2 alone ($P \leq 0.05$).

At 120 hpf, all EE2 alone treatment embryos had lower mean heart rates than the carrier control ($P \leq 0.05$). The % decrease in heart rate ranged from 7.4 % at 0.1 ng/L EE2 to 19.6 % and 18.5 % at 100 and 1000 ng/L EE2 respectively (Figure 3C). No significant difference compared to the carrier control was observed in embryos exposed to HPβCD ($P > 0.05$) (Appendix: Figure S19). The EE2 and HPβCD exposure resulted in reduced embryo heart rate at 0.1 + 1.7 ng/L, 1 + 17 ng/L, and 100 + 1700 ng/L EE2 + HPβCD treatments compared to the carrier control ($P \leq 0.05$) (Figure 3C). The reduction in heart rate of embryos exposed to EE2 and HPβCD in combination was not concentration dependent and ranged from a decrease of 7.0 % at 0.1 + 1.7 ng/L EE2 and HPβCD to a decrease of 6.8 % at 100 + 1700 ng/L EE2 + HPβCD. Furthermore, no significant difference in embryo heart rate was observed at the highest concentration of 1000 + 17000 ng/L EE2 + HPβCD. This trend differed from the EE2 alone exposure. Embryos exposed to EE2 alone had significantly reduced heart rates when compared to all EE2 + HPβCD

treatments with the exception of the 0.1 + 1.7 ng/L EE2 + HPβCD and 0.1 ng/L EE2 alone treatments ($P \leq 0.05$).

Similarly to 120 hpf, 144 hpf embryos exposed to EE2 experienced a significant decrease in heart rate at all exposure concentrations of 0.1 ng/L, 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 compared to the carrier control ($P \leq 0.05$) (Figure 3D). A maximal response was observed at 100 ng/L EE2 with a 15.0 % decrease in heart rate. Embryos exposed to 17000 ng/L HPβCD alone experienced 5.7 % decrease in heart rate compared to the carrier control ($P \leq 0.05$) (Appendix: Figure S19). No significant differences were observed in any EE2 + HPβCD treatment at 144 hpf ($P > 0.05$) (Figure 3B). In all treatments, embryos exposed to EE2 alone experienced greater reductions in heart rates when compared to EE2 + HPβCD treatments.

Embryos observed at 168 hpf did not experience significant changes in heart rates in any treatments ($P > 0.05$) (Figure 3E). However, EE2 alone had a greater reduction in heart rate at 10 ng/L compared to the 10 + 170 ng/L EE2 +HPβCD. At 192 hpf no treatments were significantly different than the control ($P > 0.05$) (Figure 3F). However, EE2 + HPβCD exposures at 0.1 + 1.7 ng and 100 + 1700 ng/L EE2 + HPβCD resulted in greater reductions in embryo heart rates compared to the corresponding EE2 alone treatment ($P \leq 0.05$) (Figure 3F).

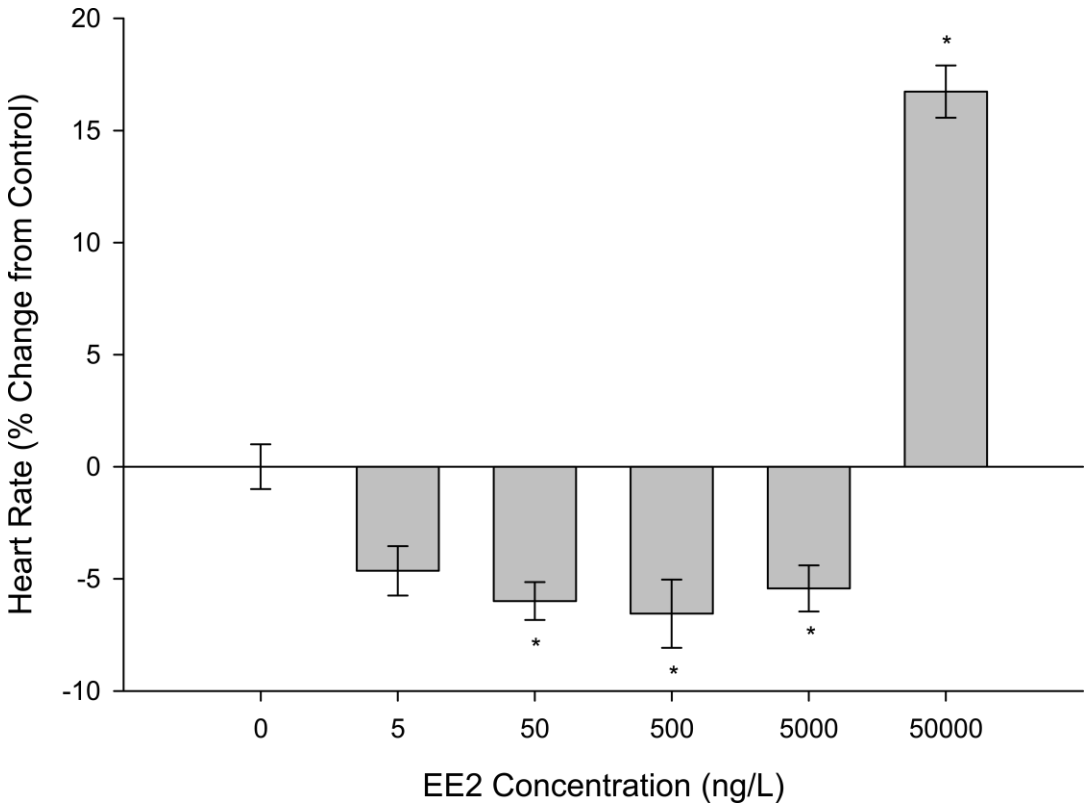


Figure 2: Pilot Study mean heart rate ± standard error (% change of control) of 120 hour post fertilization (hpf) embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2). Significant differences between treatments and control (0.01% acetone) are represented by (*) ($P \leq 0.05$), ANOVA on Ranks (Kruskal-Wallis), Tukey *post hoc*.

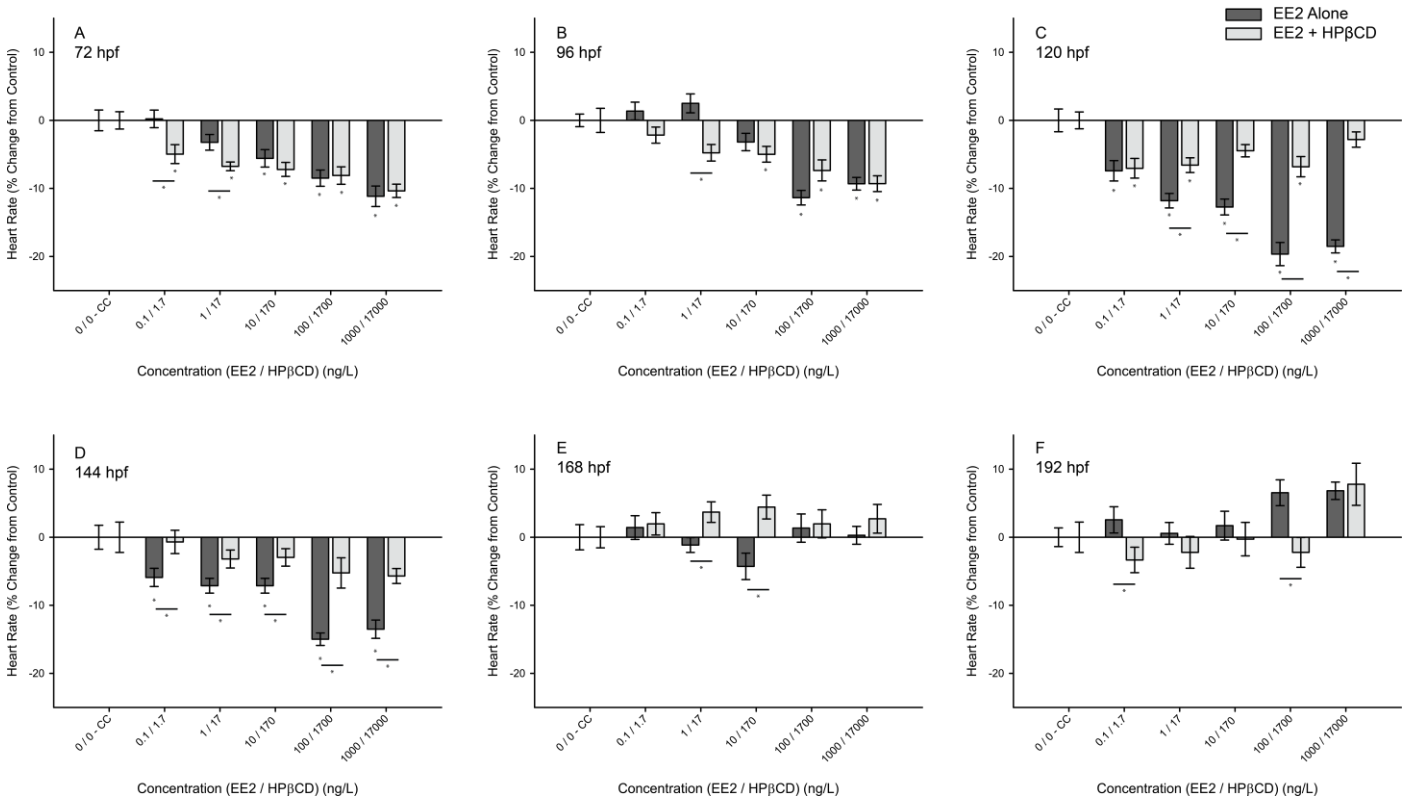


Figure 3: Range finding study mean heart rate ± standard error (% change of control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, or EE2 + hydroxypropyl-β-cyclodextrin (HPβCD). The heart rate of each embryo was assessed once every 24 hours at 72 (A), 96 (B), 120 (C): 144 (D), 168 (E), and (192) hours post fertilization (hpf). Significant differences between treatment and respective carrier controls (CC) (0.01% acetone) are represented by (*) ($P \leq 0.05$), differences between EE2 alone and EE2 + HPβCD are represented by ($\overline{\ *\ }$) ($P \leq 0.05$), Two-Way ANOVA, Dunnet's *post hoc* n=15 embryos per treatment.

2.4.2. EE2 Heart Rate Sex Comparison

Male and female embryonic Japanese medaka were exposed to nominal treatment concentrations of 0.1 ng/L to 1000 ng/L EE2. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. The heart rate of each embryo was determined once every 24 h from 120 hpf to 216 hpf, and the mean % change from control (0.01 % acetone) was calculated (Figure 4). At 120 hpf, a significant decrease in mean embryonic heart rate was observed in male embryos exposed to 100 ng/L and 1000 ng/L EE2 compared to the control ($P \leq 0.05$) (Figure 4A). Female embryos experienced a significant decrease in heart rates relative to the control following exposure to 10 ng/L, 100 ng/L and 1000 ng/L EE2 ($P \leq 0.05$) (Figure 4A). Furthermore, mean female heart rates experienced a significantly greater decrease relative to the control than males at 10 ng/L EE2 during this time point ($P \leq 0.05$) (Figure 4A).

At 144 and 168 hpf, a significant decrease in mean embryonic heart rate was observed in all treatments of 0.1 ng/L, 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 for male and female Japanese medaka relative to the control ($P \leq 0.05$) (Figure 4B, C). The decrease in heart rate appears to be concentration dependent at 168 hpf with 0.l ng/L and 1000 ng/L EE2 resulting in nearly a 5% and 12 % decrease in heart rate respectively. There was no significant difference in mean male and female heart rates at these time points ($P > 0.05$) (Figure 4B, C).

The mean embryonic heart rate of male medaka significantly decreased in all treatment concentrations of 0.1 ng/L, 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 at 192 hpf relative to the control ($P \leq 0.05$) (Figure 4D). Female medaka experienced a significant

decrease in mean heart rate at 1 ng/L, 10 ng/L, 100 ng/L, and 1000 ng/L EE2 compared to the control ($P \leq 0.05$) (Figure 4D). Mean male heart rates were significantly lower than females when exposed to 0.1 ng/L, 1 ng/L, and 100 ng/L ($P \leq 0.05$) (Figure 4D).

Measurements at 216 hpf showed a significant decrease in mean embryonic heart rates of both male and female medaka exposed to 0.1 ng/L, 100 ng/L, and 1000 ng/L EE2 ($P \leq 0.05$) Figure 4E. There were no significant differences between mean % change of male and female embryonic heart rates ($P \leq 0.05$) (Figure 4E).

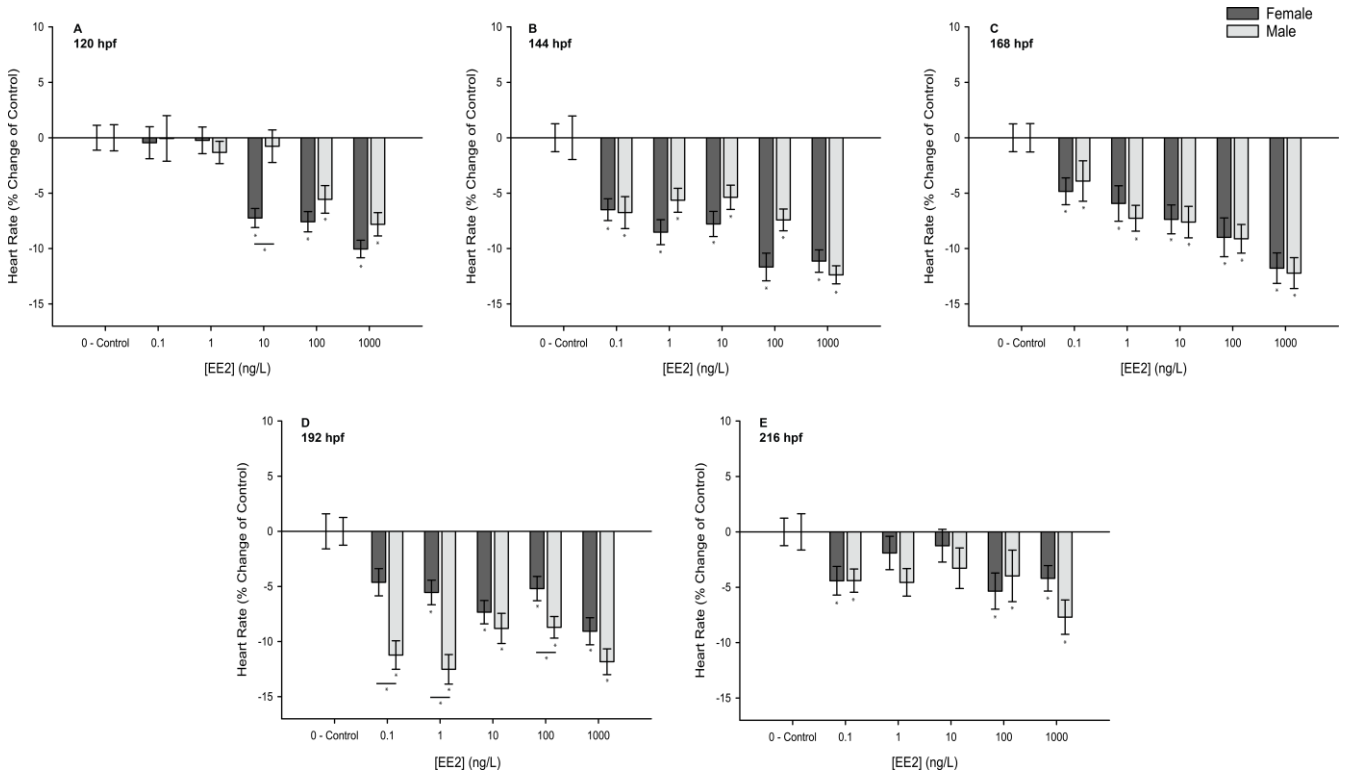


Figure 4: Mean heart rate ± standard error (% difference from control) of male and female embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone. The heart rate of each embryo was assessed once every 24 hours at 72 (A), 96 (B), 120 (C): 144 (D), 168 (E), and (192) hours post fertilization (hpf). Significant differences between treatment and respective carrier controls (CC) (0.01% acetone) are represented by (*), differences between heart rate of male and female medaka are represented by ($\overline{\ *\ }$) ($P \leq 0.05$) Two-Way ANOVA, Dunnet's *post hoc*. Each concentration was repeated three times with 7 embryos per concentration per sex.

2.4.3. ERα, ERβ, and GPER Agonists and Antagonists Impact on Embryonic Heart Rate

Mixed sex Japanese medaka embryos were exposed to EE2 alone, or in combination with an agonist or antagonist for ERα ERβ, or GPER at 24 hours post fertilization, and heart rate was determined. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. When embryonic medaka were exposed to 10 ng/L and 100 ng/L G-1, a GPER agonist, a significant decrease in mean heart rate was observed relative to the control at 120, 144, and 168 hpf ($P \leq 0.05$) (Figure 5 and Figure S20). A similar decrease in heart rate was observed in medaka exposed to 10 ng/L EE2 at these time points ($P \leq 0.05$). Exposure of medaka to EE2 and G-1 simultaneously caused a significant decrease in heart rate relative to the control ($P \leq 0.05$), although it was not significantly different from embryos exposed to either EE2 alone or G-1 alone ($P > 0.05$) (Figure 5). At 192 hpf, only embryos exposed to 100 ng/L G-1 experienced a significant decrease in heart rate ($P \leq 0.05$) (Figure S20).

Medaka embryos were exposed to Fulvestrant (ICI), a known GPER agonist and an ERα and ERβ antagonist, alone and in the presence of EE2. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. The mean heart rate of embryos exposed to 10 ng/L and 100 ng/L ICI both resulted in a significant decrease in heart rate at 120, 144, and 168 hpf relative to the control ($P \leq 0.05$) (Figure 6 and Figure S21). Similarly, exposure to 10 ng/L of EE2 resulted in a significant decrease in embryonic heart rates at all of these time points ($P \leq 0.05$) (Figure 6 and Figure S21). At 120 hpf, exposure to EE2 resulted in a decreased reduction in heart rate compared to ICI exposed embryos, however heart rate was still significantly lower than the control ($P \leq 0.05$). No significant difference

was observed when comparing EE2 and ICI alone to the simultaneous exposure of EE2 and ICI ($P > 0.05$) (Figure 6 and Figure S21). No observations at 192 hpf could be made due to a large proportion of hatched embryos at this time point.

Embryonic heart rate was monitored following the exposure of medaka embryos to G-36, a GPER antagonist, alone and in combination with EE2. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. At time points 120, 144, and 168 hpf, exposure to 10 ng/L of EE2 resulted in a significant decrease in heart rate ($P \leq 0.05$) (Figure 7 and Figure S22). There was no significant change in heart rate of embryos exposed to 10 ng/L and 100 ng/L of G-36 ($P > 0.05$) (Figure 7 and Figure S22). Additionally, embryos exposed to a combination of EE2 and G-36 experienced no change in heart rate relative to the control ($P > 0.05$) (Figure 7 and Figure S22). No significant change in heart rate from the control at 192 hpf occurred ($P > 0.05$) (Figure 7 and Figure S22).

The heart rate of embryonic medaka was observed following exposure to PHTPP, an ERβ antagonist and EE2. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. At 120, 144 and 168 hpf, a decrease in heart rate was observed in embryos exposed to 10 ng/L EE2 relative to the control ($P \leq 0.05$) (Figure 8 and Figure S23). Conversely, at 120 and 144 hpf, heart rate of embryos exposed to 10 ng/L and 100 ng/L PHTPP did not differ from the control ($P > 0.05$) (Figure 8 and Figure S23). At 168 hpf exposures of 10 ng/L and 100 ng/L PHTPP resulted in a significant decrease in embryo heart rates compared to the control ($P \leq 0.05$), but this effect was still significantly different from the EE2 alone treatment ($P \leq 0.05$) (Figure 8 and Figure S23). At 120, 144 and 168 hpf, embryos exposed to EE2 and PHTPP at the same time experienced a significant

decrease in hear rate relative to the control ($P \leq 0.05$) (Figure 8 and Figure S23). Due to a large proportion of hatching, heart rate was unable to be determined at 192 hpf.

Finally, embryonic medaka were exposed to an ERα antagonist, ZK 164015. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. At 120, 144, 168 hpf a significant decrease in heart rate relative to the control was observed following exposure to 10 ng/L EE2 alone and when combined with ZK 164015 ($P \leq 0.05$) (Figure 9 and Figure S24). The heart rate of embryos exposed to ZK 164015 alone did not differ from the control at these time points ($P \leq 0.05$) (Figure 9 and Figure S24). No observations were made at 192 hpf due to the high proportion of hatched embryos.

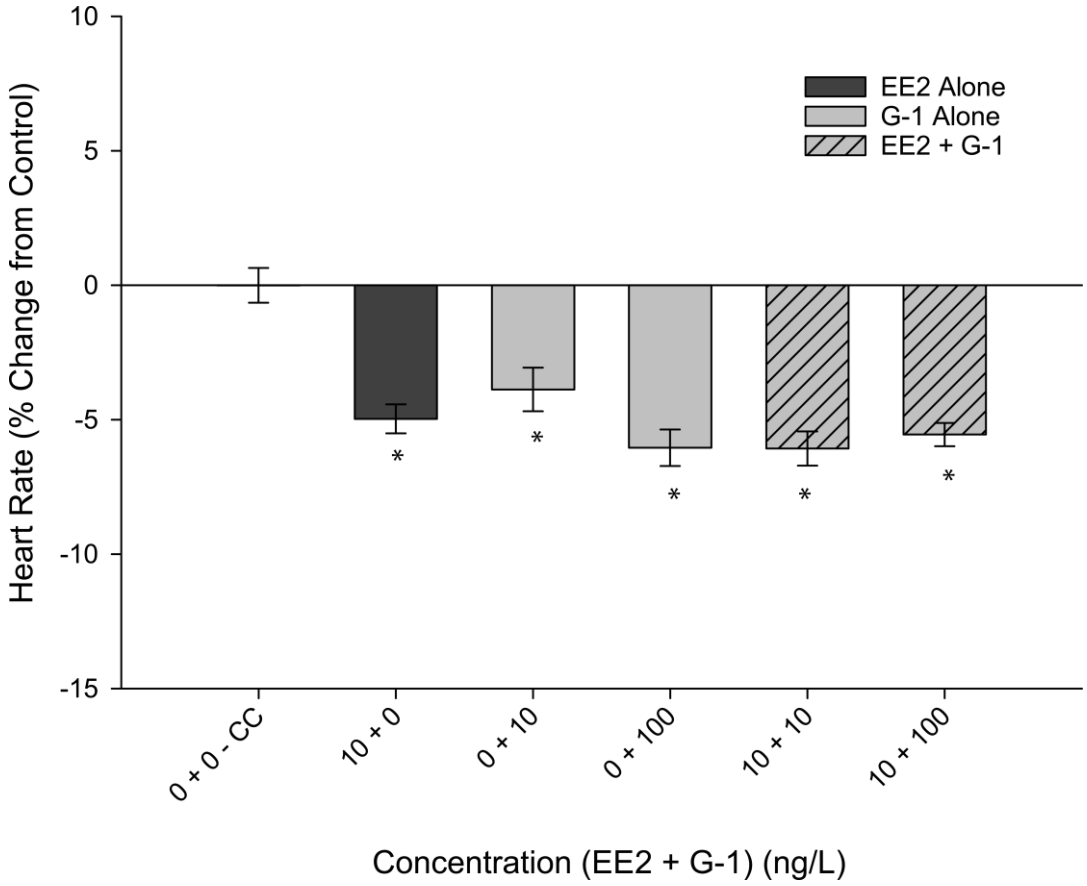


Figure 5: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, G-1, a G Protein-Coupled estrogen receptor (GPER) agonist alone, or EE2 + G-1. Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA on Ranks (Kruskal-Wallis), Tukey *post hoc*.

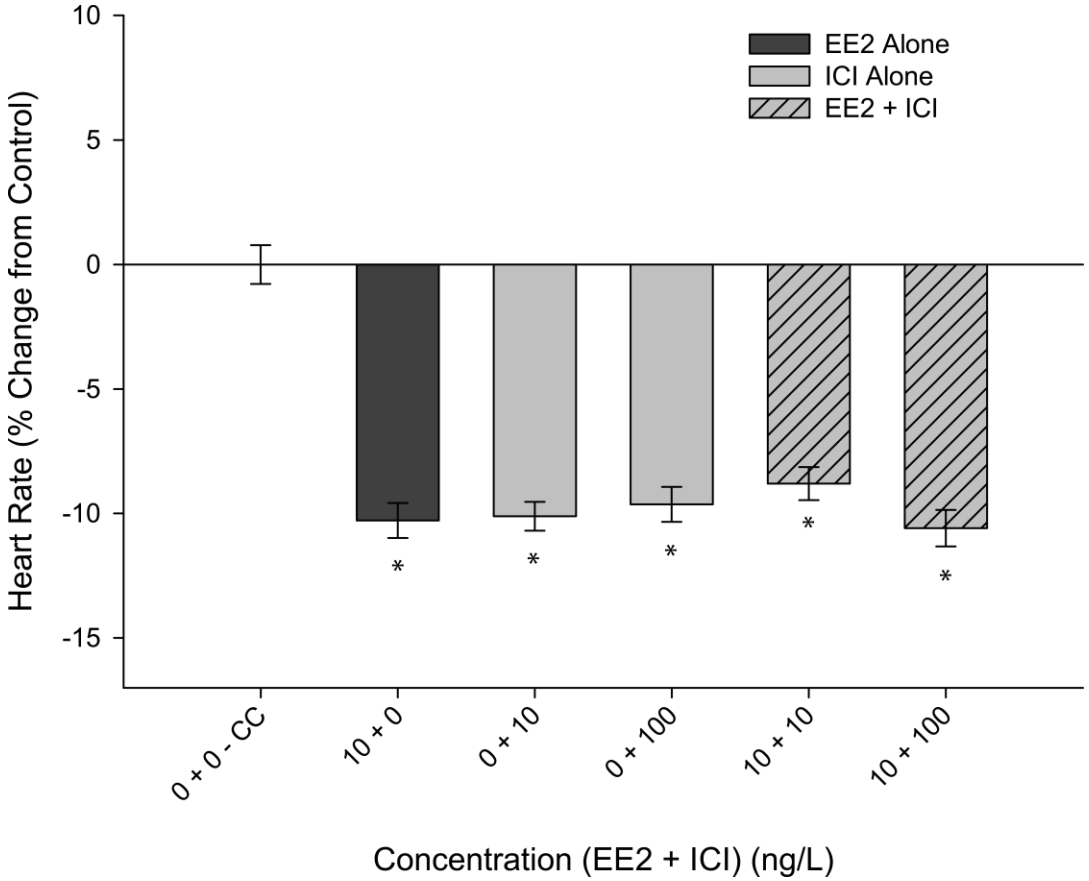


Figure 6: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, Fulvestrant (ICI) (GPER agonist and ERα and ERβ antagonist) alone, or EE2 + ICI. Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) (P≤0.05), ANOVA, Tukey *post hoc*.

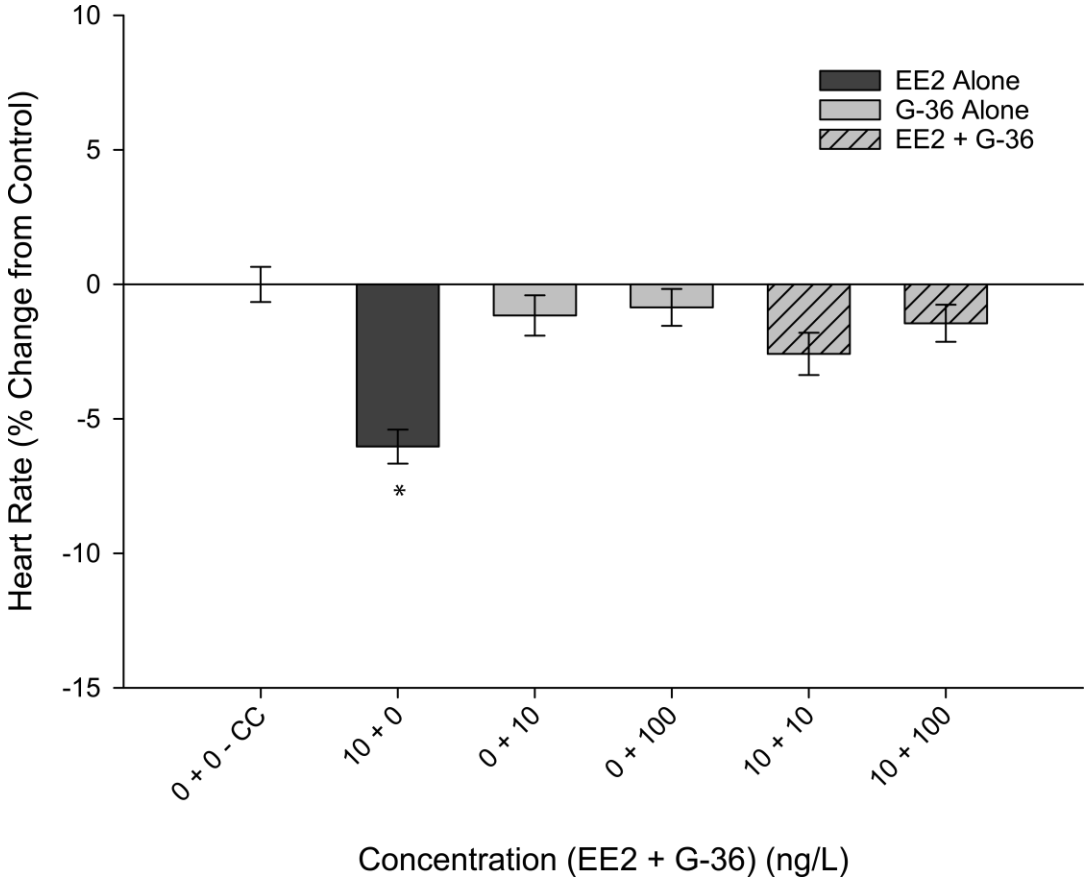


Figure 7: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, G-36 (GPER antagonist) alone, or EE2 + G-36. Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA, Tukey *post hoc*.

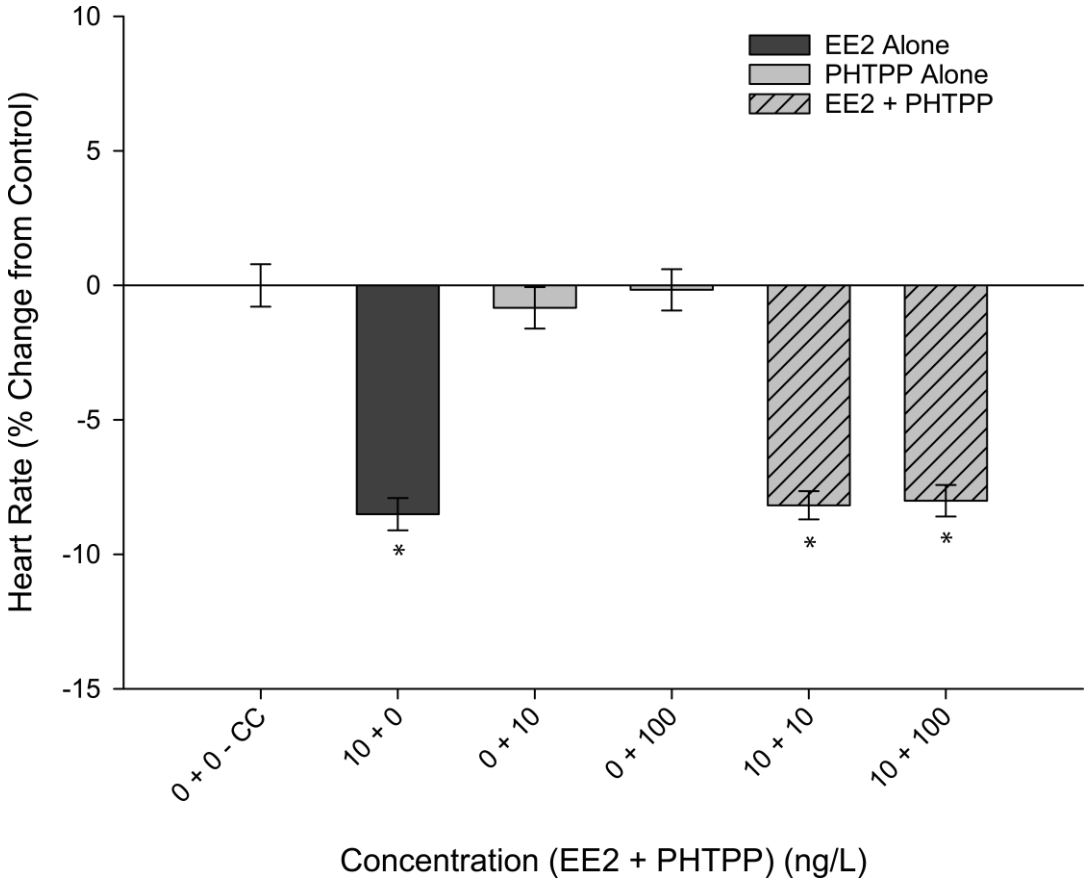


Figure 8: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, PHTPP (ERβ antagonist) alone, or EE2 + PHTPP. Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA on Ranks (Kruskal-Wallis), Tukey *post hoc*.

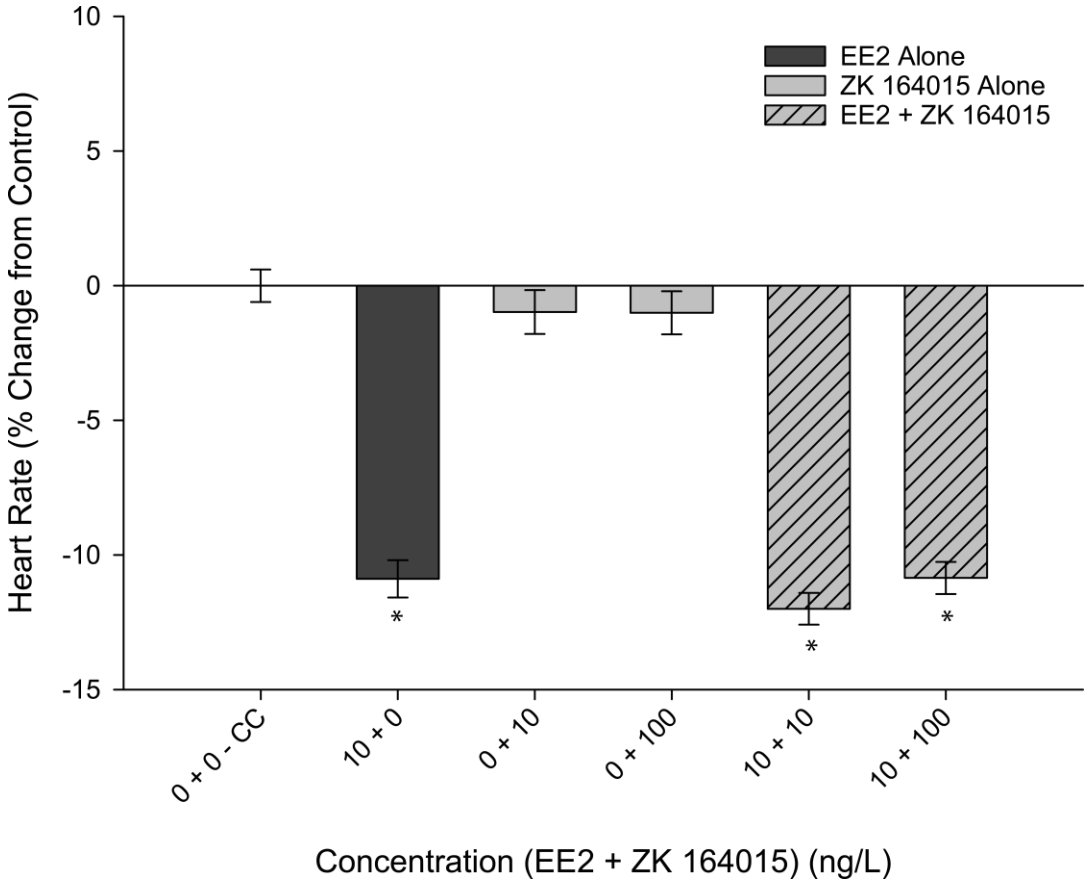


Figure 9: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, ZK 164015 (ERα antagonist) alone, or EE2 + ZK 164015. Each treatment consists of three replicates containing 7 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA on Ranks (Kruskal-Wallis), Tukey *post hoc*.

2.4.4. The Influence of HPβCD on EE2 Toxicity

In order to understand the influence that HPβCD has on the embryo toxicity of EE2, mixture studies were conducted, and heart rate was assessed. Temperature was monitored and maintained at 27.0 ± 0.5° C throughout all treatments. As observed in previous sections, exposure of medaka embryos to EE2 alone resulted in decreased heart rates at 10 ng/L at 120, 144 and 168 hpf ($P \leq 0.05$) (Figure 10 and Figure S25). No significant differences were observed in the heart rate of medaka embryos exposed to 180 ng/L of HPβCD ($P > 0.05$) (Figure 10 and Figure S25). However, when EE2 was combined with HPβCD, a reduced effect was observed, meaning the heart rate became more similar to the control as the concentration of HPβCD increased. At 120, 144, and 168 hpf there were no significant differences in heart rates between the control and embryos exposed to 10 ng/L EE2 and 188 ng/L HPβCD , a 1:4 molar ratio ($P \leq 0.05$) (Figure 10 and Figure S25). Furthermore, at 144 hpf there were no significant differences in embryo heart rates between the control and 10 ng/L EE2 + 94 ng/L HPβCD, a 1:2 molar ratio ($P \leq 0.05$) (Figure 10). Heart rate at 192 hpf was unable to be measured due to a high proportion of hatched embryos.

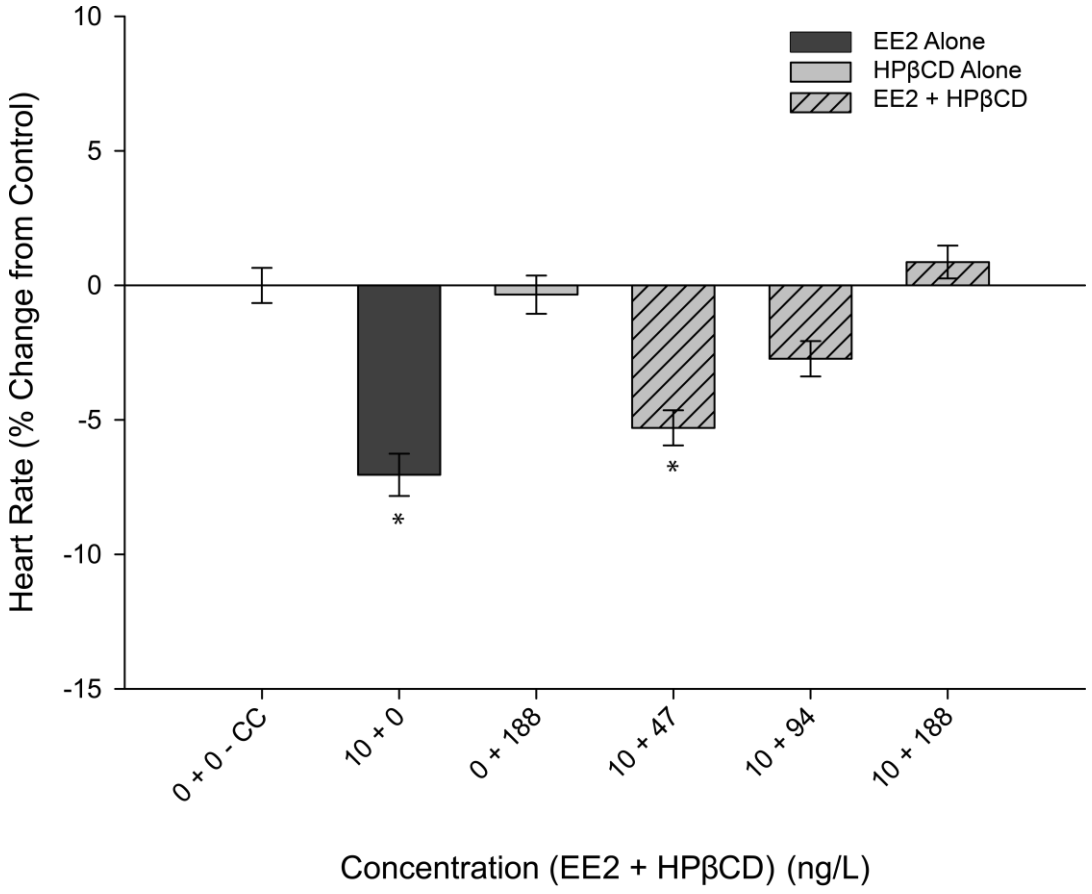


Figure 10: Mean heart rate ± standard error (% difference from control) of 144 hpf embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, hydroxypropyl-β-cyclodextrin (HPβCD) alone, or EE2 + HPβCD in a 1:1 (10 ng/L + 47 ng/L), 1:2 (10 ng/L + 94 ng/L), and 1:4 (10 ng/L + 188 ng/L) molar radio. Each treatment consists of three replicates containing 7 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA on Ranks (Kruskal-Wallis), Tukey *post hoc*.

2.5. Discussion

The environmental presence of pharmaceuticals and personal care products (PPCPs) has been an area of intrigue in the field of aquatic toxicology. Few PPCPs have been investigated as extensively as EE2, with many studies demonstrating high potency in non-target organisms (Parrott and Blunt, 2005; Kidd *et al.*, 2007; Bhandari *et al.*, 2015). This toxicity has often been related to ERα and ERβ activation, known as classical estrogen signaling, usually leading to feminization of males, and alterations in fertility and fecundity in fish (Jayasinghe and Volz, 2012; Prossnitz and Barton, 2014; Overturf *et al.*, 2015; Romano *et al.*, 2017). These effects have been demonstrated both in the laboratory and in whole lake systems (Parrott and Blunt, 2005; Kidd *et al.*, 2007; Blanchfield *et al.*, 2015). More recently, there has been a greater interest in non-classical estrogen signaling in non-target organisms. This was aided by the discovery that estradiol (E2) is the ligand for the G protein-coupled estrogen receptor (GPER), formerly G protein receptor 30 (GPR30) (Jayasinghe and Volz, 2012; Romano *et al.*, 2017).

GPER is localized intracellularly on the membrane of the sarcoplasmic reticulum (Filardo and Thomas, 2012; Prossnitz and Barton, 2014; Barton, 2016; Sharma *et al.*, 2018). Expression of GPER (*gper1*) occurs throughout the body of vertebrates with higher levels found in the gonad, nervous system, and vasculature (Jayasinghe and Volz, 2012). Exposure of embryonic fish to estrogens and estrogen mimics has resulted in altered heart rates. Zebrafish (*Danio rerio*) embryos exposed to 1 mg/L estradiol (E2) experienced an increase in heart rate, tachycardia, while a decrease, bradycardia, was observed in embryonic zebrafish exposed to 0.4 ng/L 17α-ethinylestradiol (EE2) (Santos *et al.*, 2014; Romano *et al.*, 2017). Romano *et al.*, (2017) demonstrated that GPER activation was

responsible for the increase in embryonic zebrafish heart rate. GPER activation presents a novel, non-classical, mode of action for EE2 toxicity in embryonic fish.

A goal of this chapter was to determine how EE2 impacts heart rates of embryonic Japanese medaka at environmentally relevant concentrations. We also wanted to identify if the odour suppressant/excipient hydroxypropyl-β-cyclodextrin (HPβCD) could alter the observed toxicity of EE2.

The first experiment in this chapter was completed to determine the general effect of EE2 on embryonic Japanese medaka heart rate. At 120 hours post fertilization (hpf), a decrease in heart rate was observed at 50 ng/L and an increase at 50000ng/L EE2 (Figure 2). These bimodal results were consistent with the previous research where low and high concentrations of estrogenic compounds resulted in bradycardia and tachycardia, respectively (Santos *et al.*, 2014; Romano *et al.*, 2017). Furthermore, this demonstrated that the heart rate of FLFII Japanese medaka respond similarly to zebrafish, when exposed to EE2. It would be beneficial to further assess species differences in the future. The range finding study was conducted to identify the potential for HPβCD to alter this observed effect and establish a more accurate effective range for EE2 at environmentally relevant concentrations. It was decide to test lower concentrations of EE2, due to the observed effect at 5 ng/L in the pilot study and the to determine if we could replicate the effects observed by Santos *et al.*, (2014). In this study, the most apparent effect was observed at 120 and 144 hpf with a decrease was observed at 0.1 ng/L EE2 (Figure 3). This effect was mitigated by the presence of HPβCD, with the heart rate at control levels (Figure 3). These results provided justification for investigating the mode of action of EE2 and how HPβCD disrupts this toxicity at environmentally relevant concentrations.

The pilot study demonstrated that embryonic heart rate may be a valuable endpoint for monitoring EE2 toxicity in embryonic medaka. Based on previous research it was speculated that these effects were the result of GPER activation (Romano *et al.*, 2017). Given that the natural ligand for GPER is E2, it was speculated that differential expression of *gper* in male and female embryos may occur. Since sex can be determined as early as 72 hpf in female leucophore free (FLFII) medaka, it was decided that sex specific toxicity should be investigated prior to conducting other experiments. It was also unknown if the control heart rate differs between male female medaka embryos, which provided additional justification for this experiment.

Following exposure, few differences in heart rate were observed between male and female embryonic heart rates, with the exception of the 192 hpf measurment (Figure 4). Moving forward from this study, embryos were exposed at 24 hpf rather than 6 hpf to ensure that there were a sufficient number of embryos in each replicate well. Unfertilized medaka embryos become blue in colour between 6 and 24 hpf when in rearing solution. Although some differences were observed between sexes at 192 hpf, both showed a similar response to EE2 exposure, and control heart rates were statistically similar. Thus, it was concluded that sexes did not require separation for future experimentation. Although separation by sex was not conducted in subsequent studies, future research in this area may be beneficial for explaining some of the variability observed, particularly when nearing hatch. The similarities in response were not surprising due to the expression of *gper* in many male tissues (Liu *et al.*, 2009; Prossnitz and Barton, 2014). It has also been demonstrated that some *gper* mRNA is deposited into the embryo through maternal transfer (Jayasinghe and Volz, 2012). The presence of maternally transferred *gepr* mRNA may

offset any differential expression in males and females during embryo development. Sex dependent toxicity may become more apparent during life stages that are beyond embryogenesis; this was not studied in this thesis. It should be noted that the observed bradycardia appeared to be somewhat transient since the effect became less apparent as the embryos approached hatching (Figure 4). This may be indicative of the development of uptake/elimination as well as detoxification pathways (Hart, 1983; Iwamatsu, 2004; Kais *et al.,* 2013). Uptake and elimination of EE2 during embryogenesis is examined in the following chapter.

Although Romano *et al.,* (2017) demonstrated that GPER activation was responsible for high concentration E2 induced tachycardia, the mode of action for low level EE2 induced bradycardia has not yet been elucidated. In this chapter, embryonic medaka were exposed to a series of estrogen receptor agonists and antagonists. G-1 was the first compound that was studied, a known selective GPER agonist in vertebrates. A similar response to EE2, decreased heart rate, was observed (Figure 5). This indicted that GPER activation can be, at least partly, responsible for the observed bradycardia. The next compound tested was Fulvestrant (ICI), a GPER agonist and an ERα and ERβ antagonist. The goal of this exposure was to further isolate GPER, while blocking EE2 activation of an ERα and ERβ. Comparable to G-1, ICI resulted in a similar response to EE2 alone (Figure 6). This experiment helped further demonstrate that GPER, not classical estrogen signaling, was responsible for the observed bradycardia.

Next, we antagonized GPER with G-36, a known selective GPER antagonist. This exposure resulted in no change in heart rate from the control when alone or in combination with EE2 (Figure 7). This demonstrated that when GPER is antagonized, EE2 cannot

induce bradycardia in embryonic medaka. Finally, we examined PHTPP and ZK 164015, ERβ and ERα antagonists respectively. PHTPP alone at 120 and 144 hpf resulted in no change in heart rate ($P > 0.05$). However, at 168 hpf a decrease was observed relative to the control ($P \leq 0.05$), this decrease was significantly less than EE2 alone ($P \leq 0.05$). In both cases, PHTPP and ZK 164015 alone resulted in no change in heart rate, while exposure with EE2 resulted in a typical reduction of heart rate (Figure 8). This demonstrated that antagonizing ERα and ERβ individually had no impact on the ability of EE2 to cause reduced heart rate. In summary, these results are consistent with those of Romano *et al.* (2017). In their study, an increase was observed at 1 mg/L E2. The results found in our study demonstrate that the activation of GPER is responsible for the observed bradycardia in embryonic Japanese medaka.

Localization of GPER and downstream signaling was investigated by Romano *et al.* (2017). They demonstrated that at high concentration of E2, GPER activation in the pituitary, upstream of triiodothyronine (T3) signaling can increase embryonic heart rate (Romano *et al.*, 2017). Furthermore, tachycardia of *gper* mutants was rescued by T3 and not E2 (Romano *et al.*, 2017). This indicates that T3 acts downstream of GPER in embryonic heart rate regulation.

Given these results, there still exists the possibility that E2 could be altering intracellular calcium concentrations. Cytoplasmic calcium primarily originates from the sarcoplasmic reticulum within the cell (Evans *et al.*, 2013; Vornanen, 2016). The G protein-coupled estrogen receptor (GPER) is localized on the sarcoplasmic reticulum, and activation results in increased intracellular cellular calcium levels (Diamante *et al.*, 2017). This could result in two additional potential mechanisms of action. Firstly, the membrane

could become more positive, thus increasing the plateau phase in ventricular myocyte depolarization, or in pacemaker cells such that rhythmic depolarization reaches the threshold of action potential more easily. Secondly, by occupying the *TnC* binding site on troponin, calcium allows myosin to bind to actin initiating the cross-bridge reaction, resulting in cardio myocyte contraction (Bers, 2008). Conversely, relaxation occurs when the membrane is repolarized and calcium returns to resting levels (Bers, 2008). Therefore, an increased intracellular calcium concentration could potentially lead to an extension of the contraction phase of cardiac myocytes; this may result in slowing the cardiac cycle, inducing bradycardia. This is a hypothesis that was not tested in our study, or the study by Romano *et al.,* (2017). However, this potential mode of action may explain why different responses were observed at high concentration of E2 in the Romano *et al.,* (2017) study, and at the low concentrations of EE2 in this, and the study conducted by Santos *et al.,* (2017).

The next experiment that was completed in this chapter examined the influence of HPβCD on the toxicity of EE2. Both EE2 and HPβCD are thought to exist together in surface water via municipal wastewater effluent. It has been previously noted that EE2 can be included, non-covalently, in the binding cavity of HPβCD (Pérez and Escandar, 2013). It has also been shown that HPβCD can suppress the estrogenic effects of E2 in a yeast two-hybrid assay (Oishi *et al.,* 2008). HPβCD had no impact on E2 estrogen receptor affinity, suggesting that HPβCD may be blocking E2 from crossing biological membranes (Oishi *et al.*, 2008). Given that GPER, like ERα and ERβ, is located inside the cellular membrane, the bulky, hydrophilic nature of HPβCD impedes EE2 from interacting with

GPER. In our study the additional barrier of the chorion must also be overcome to achieve receptor activation.

We exposed medaka embryos across embryogenesis to 10 ng/L EE2 and a 1:1, 1:2, and 1:4 ratio of EE2:HPβCD. It has been shown that HPβCD has optimal guest inclusion at a ratio of 1:4 (Loftsson and Brewster, 2012). Our result demonstrated that at a 1:4 ratio of EE2:HPβCD resulted in a reduction of bradycardia in embryonic Japanese medaka when compared to EE2 exposed embryos (Figure 10). Furthermore, the heart rate of embryos exposed to 1:4 EE2:HPβCD were not statistically different from the control. A trend of lowered heart rate, relative to the control, was also observed as HPβCD concentrations were reduced. These results indicate that HPβCD may be interacting with EE2 and limiting its ability to interact with GPER.

Overall, this chapter demonstrated that EE2 can induce bradycardia in embryonic Japanese medaka. It was also demonstrated that this is the result of GPER activation, non-classical estrogen signaling. This is an important finding in the field of aquatic toxicology because it provides an additional mechanism of action for a very well-studied toxicant, EE2. Our results also demonstrated that HPβCD could reduce the effect of EE2 on embryonic Japanese medaka heart rate, highlighting a novel interaction in this field. The following chapters will investigate the uptake, elimination, and metabolic rate of embryos exposed to EE2 in the presence and absence of HPβCD.

Chapter 3: Embryonic Uptake and Elimination of EE2, and the Influence of HPβCD

3. Chapter 3: Embryonic Uptake and Elimination of EE2, and the Influence of HPβCD

3.1. Introduction

In Chapter 2, it was demonstrated that 17α-ethinylestradiol (EE2) could reduce the heart rate of embryonic Japanese medaka (*Oryzias latipes*) at environmentally relevant concentrations. These results were supported by Santos *et al.*, 2014, exposing 0,4 ng/L of EE2 to embryonic zebrafish. It was also demonstrated that these effects were most likely due to the activation of the G protein-coupled estrogen receptor (GPER), a finding supported by the results of Romano *et al.*, (2017). Decreased heart rate was observed in all treatments involving a GPER agonist at 120 and 144 hours post fertilization (hpf). However, as embryogenesis progressed, this effect often disappeared. The heart rate often returned back to control levels as hatching approached, while still in the presence of the agonist. This observation raised questions regarding the uptake and elimination of EE2 from the embryo. The permeability of the chorion of fish has been known to increase as development progresses, but the elimination of certain contaminants during this time period is understudied (González-Doncel *et al.*, 2004). Identifying the uptake and elimination of EE2 through the use of a radioisotope was thus one of the major goals of this research.

Another important goal was to understand the effects of hydroxypropyl-β-cyclodextrin (HPβCD) on EE2 uptake. Understanding the toxicity of mixtures has become increasingly important to aquatic toxicologists. Although the toxicity of multiple compounds can be extremely difficult to discern, it is extremely relevant when trying to replicate the complex mixtures found in wastewater. Mixtures involving HPβCD are very

intriguing due to its ability to chemically interact with guest compounds. In Chapter 2, it was demonstrated that in the presence of HPβCD, EE2 was no longer able to reduce the embryonic heart rate of Japanese medaka at 120 – 168 hpf. A hypothesis for this decreased toxicity is that HPβCD included EE2, increasing its bulkiness and inhibiting its ability to cross cellular membranes and potentially the chorion. If the HPβCD-EE2 complex was able to enter the chorion, it might still be impeded from being taken up by the developing embryo itself. Thus, the second major goal of this research was to identify the role of HPβCD in the uptake of EE2 into Japanese medaka embryos.

3.2. Objectives, Knowledge Gaps, Rationale, and Hypotheses

The overall goal of this chapter was to determine the uptake of ^{14}C-EE2 in embryonic Japanese medaka in the presence and absence of HPβCD and to help answer two major questions. Firstly, how readily is EE2 taken up and eliminated by embryonic Japanese medaka and does uptake and elimination of EE2 occur uniformly across embryogenesis? Secondly, does HPβCD alter the uptake of EE2? This study was aimed at aiding in the understanding of how HPβCD may modify guest toxicity. Since HPβCD is thought to not readily cross biological membranes (Loftsson and Brewster, 2012), reduced EE2 uptake across the chorion may be the result of inclusion with HPβCD.

3.2.1. Objective 1: ^{14}C-EE2 Rangefinder Assay

The goal of objective 1 was to determine a suitable exposure concentration range of ^{14}C-EE2 such that the amount taken up by the embryo can be detected by the scintillation counter. This objective provided an appropriate exposure concentration of ^{14}C-EE2 for the remaining objectives in this chapter.

The null hypothesis for this experiment was:

H_0: The amount of ^{14}C-EE2 taken up by embryonic Japanese medaka exposed to ^{14}C-EE2 was not significantly different from the control group.

3.2.2. Objective 2: ^{14}C-EE2 Uptake and Elimination Assay

Objective 2 was examined in order to determine uptake of ^{14}C-EE2 throughout embryogenesis. It was observed in Chapter 2 that the effect of decreasing embryonic heart

rate appears to dissipate as the embryos approach hatch (192-216 hpf). This finding raised questions about the uptake and elimination of EE2 as embryogenesis progresses towards hatch. As development occurs, many physiological processes begin to initiate, including alterations in the permeability and elimination of toxicants from the embryo (González-Doncel *et al.*, 2004; Adams *et al.*, 2005). This experiment examined various lengths of exposure and depuration following exposure to ^{14}C-EE2.

The null hypotheses for this experiment were:

H_0: The amount of ^{14}C-EE2 taken up by embryonic Japanese medaka was not significantly different than the control at any time point tested.

H_0: The amount of ^{14}C-EE2 taken up by embryonic Japanese medaka was not significantly different between any time points sampled.

3.2.3. Objective 3: The Influence of HPβCD on the Uptake of ^{14}C-EE2

The null hypothesis for this experiment was:

H_0: The amount of ^{14}C-EE2 within Japanese medaka embryos exposed to ^{14}C-EE2 will not be different than those exposed to the carrier control (0.01 % acetone).

H_0: The amount of ^{14}C-EE2 within Japanese medaka embryos exposed to ^{14}C-EE2 will not be different than embryos exposed to ^{14}C-EE2 + HPβCD.

3.3. Materials and Methods

3.3.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. 0.1mCi/ml ^{14}C-EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol) in ethanol with a specific activity of 55 mCi/ml, was purchased from American Radiolabeled Chemicals, Inc. (*St. Louis, Missouri, USA*). Hydroxypropyl-β-cyclodextrin (HPβCD), 97.0 %, purchased from Acros Oganics (*New Jersey, USA*) was dissolved in deionized water. Treatment solution were made using rearing solution as a diluent. An acetone concentration of 0.01 % v/v was maintained in each treatment as a carrier and for consistency from previous experiments.

3.3.2. Scintillation Counter and Calculations

Determination of EE2 uptake was completed using a TriCarb Liquid Scintillation Analyzer (PerkinElmer, *Waltham, Massachusetts, United States*) and radiolabeled EE2 (^{14}C-EE2). The ionizing radiation from ^{14}C-EE2 is detected by the scintillation counter by measuring the light pulses caused by the excitation of incident radiation in the scintillation fluid. This is reported in counts per minute and converted based on machine efficiency to disintegrations per minute (DPM) (Equation 2). DPM was then be converted to microcuries (µCi) (Equation 3). A blank consisting of scintillation fluid only was used and subtracted from the count. Next, µCi was converted to the number of moles of ^{14}C-EE2 using the

specific activity of ^{14}C-EE2 (Equation 4). Finally, the mass of ^{14}C-EE2 was determined (Equation 5); this represents the amount of ^{14}C-EE2 taken up by the organism.

$$DPM = \frac{CPM}{Efficiency}$$

Equation 2: Conversion of counts per minute (CPM) to disintegrations per minute (DPM). Efficiency is calculated using a quench curve and PerkinElmer QuantaSmart (*Waltham, Massachusetts, United States*).

$$x = \frac{(DPM - blank)}{2220000}$$

Equation 3: Conversion of disintegrations per minute (DPM) to µCi, where x represents the number of µCi, and 2220000 is the number of disintegrations per minute in a µCi.

$$i = \left(\frac{x}{1000}\right)(y^{-1})$$

Equation 4: Conversion of the number of µCi to moles, where *i* represents the number of moles, *x* represents the number of µCi determined in Equation 3, and *y* represents the specific activity of the radioisotope used.

$$\alpha = i \left(296.403 \frac{g}{mol}\right)$$

Equation 5: Conversion of moles to mass in grams, where α represents the mass in grams, *i* represents the number of moles determined in Equation 4, and 296.406 g/mol is the molar mass of EE2.

3.3.2.1. Instrument Calibration, Normalization, Performance Assessment, and Quench

Calibration of the instrument ensures the photomultiplier tube response are synchronized to a Carbon-14 standard, resulting in accurate energy quantification of all beta particle emissions. Normalization establishes a numerical scale for sample quench. An unquenched Carbon-14 standard was counted and assigned a value of 1000 to the lowest allowable value for the Quench Indicating Parameter, tSIE (transformed Spectral Index of the External Standard). This allowed numerical values to be assigned to sampled quenches. Instrument performance assessment ensured that the instrument was operating within acceptable ranges of background counts, counting efficiency, sensitivity, and reproducibility. The background counts from the blank, containing scintillation fluid and dilution water, was subtracted from all sample counts (PerkinElmer, 2015).

Calibration, normalization, and instrument performance assessment occurs automatically in the TriCarb Liquid Scintillation Analyzer (PerkinElmer, *Waltham, Massachusetts, United States*). A Self-Normalizing and Calibration (SNC) cassette, and an Instrument Performance Assessment (IPA) cassette from PerkinElmer (*Waltham, Massachusetts, United States*) were used. These cassettes remained on the counting deck at all times and contain a Carbon-14 calibration standard, an unquenched Tritium standard, and a background standard. Calibration, normalization and an instrument performance assessment was conducted prior to sample analysis unless it had been completed within the previous 23 hours (PerkinElmer, 2015).

A quench curve was used to correlate the sample counting efficiency of the instrument to a quench indicating parameter. Chemical and colour quench may occur, and can reduce counting efficiency of a sample due to the interference of production or detection of the photons produced when radioactive emissions interact with the scintillation solution. A quench standards assay was completed using quench standards from PerkinElmer (*Waltham, Massachusetts, United States*). The quench standards contain the same amount of nuclide with varying amounts of quenching agent, and the program QuantaSmart generated a quench curve to be stored it in the quench standards library. The quench curve was used to determine the counting efficiency of the instrument and calculated the disintegrations per minute (DPM) (Equation 2).

3.3.3. Exposure Conditions

Exposure of medaka embryos occurred in a fume hood within an approved radioisotope laboratory at the University of Ontario Institute of Technology (*Oshawa, Ontario, Canada*). The 6 well plates were placed on top of a heating pad in order to maintain similar temperatures (~27° C) to those achieved in previous experiments. The photoperiod was maintained at 16h light and 8 hours dark with a full spectrum LED lightbulb and an analogue timer. Solution changes and temperature monitoring occurred daily.

3.3.4. Sample Analysis

Sample detection occurred using a Tri-Carb 2800TR Liquid Scintillation Analyzer and analyses were completed using QuantaSmart software (PerkinElmer, *Waltham,*

Massachusetts, United States). After exposure to ^{14}C-EE2, embryos were rinsed first with unlabeled EE2, and then rinsed 5 times with clean lab water to remove any ^{14}C-EE2 that may have been residing on the outside of the chorion. All embryos from each replicate were then placed in 5 mL disposable scintillation vials containing 5 mL of Ultima Gold LCS Cocktail Sigma-Aldrich (*Oakville, Ontario, Canada*). All embryos per replicate were required to be pooled for analysis to achieve a strong enough signal for detection. Sample analysis occurred for 1 minute per sample, and the amount of ^{14}C-EE2 was determined using Equation 2-5.

3.3.5. Objective 1: Rangefinder Assay

Embryonic Japanese medaka were obtained as described in section 1.7.1.2, and maintained as described in section 3.3.3. At 24 hpf, embryos were exposed to 10 mL of 0 ng/L (Control), 100 ng/L, 1000 ng/L or 10000 ng/L ^{14}C-EE2 in a 6 well plate Each treatment was repeated 3 times with 40 embryos per replicate, (Table 8) and at 120 hpf, the embryos were sampled as described in section 3.3.4 above.

Table 8: Exposure summary for Objective 1: Rangefinder Assay. Concentrations of ^{14}C-17α-ethinylestradiol (^{14}C-EE2), which embryonic Japanese medaka (*O. latipes*) were exposed to, the number of embryos per treatment, and the number of replicates.

[^{14}C-EE2] (ng/L)	Number of Embryos	Replicates
0 – Control	40	1
100	40	1
1000	40	1
10000	40	1

3.3.6. Objective 2: EE2 Uptake and Elimination Assays

3.3.6.1. Uptake

Embryonic Japanese medaka were obtained as described in section 1.7.1.2, and maintained as described in section 3.3.3. For the continuous exposure study, 60 embryos per replicate were used rather than 40 to ensure a strong enough signal in the scintillation counter. Embryos were exposed to 10 mL of 10 µg/L ^{14}C-EE2 in a 6 well plate for varying lengths of time. All exposures began at 6 hpf, and sampling occurred at 24, 48, 96, and 168 hpf (Table 9). Sampling was conducted as described in section 3.3.4 above. A non-linear regression and an ANOVA with a Tukey *post* hoc test were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*).

Embryonic Japanese medaka were obtained as described in section 1.7.1.2, and maintained as described in section 3.3.3. For the pulse exposure study, embryos were exposed to 10 mL of 10 µg/L ng/L ^{14}C-EE2 in a 6 well plate for 24 hours. All exposures began at 6, 24, 48, 72, 96, 120, or 144 hpf, and were sampled 24 hours after exposure began as described in section 3.3.4 above. Three replicates per exposure window and 60 embryos per replicate were used (Table 10). A non-linear regression and an ANOVA with a Tukey *post* hoc test were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*).

Table 9: Exposure duration of Japanese medaka (*O. latipes*) to ^{14}C-EE2, Objective 2: Uptake, continuous exposure. The darkened line ▬ represents the time of exposure to 10 µg/L ^{14}C-EE2, and "Sampled" denotes the time point when the embryos were analyzed in hours post fertilization (hpf).

	Hours Post Fertilization (hpf)							
Test #	6	24	48	72	96	120	144	168
1	▬	Sampled						
2	▬	▬	Sampled					
3	▬	▬	▬	▬	Sampled			
4	▬	▬	▬	▬	▬	▬	▬	Sampled

Table 10: Exposure duration of Japanese medaka (*O. latipes*) to ^{14}C-EE2, Objective 2: Uptake, pulse exposure study. The darkened line ▬ represents the time period of pulse exposure to 10 µg/L ^{14}C-EE2, and "Sampled" denotes the time point when the embryos were analyzed in hours post fertilization (hpf).

	Hours Post Fertilization (hpf)							
Test #	6	24	48	72	96	120	144	168
1	▬	Sampled						
2		▬	Sampled					
3			▬	Sampled				
4				▬	Sampled			
5					▬	Sampled		
6						▬	Sampled	
7							▬	Sampled

3.3.6.2. Elimination:

Embryonic Japanese medaka were obtained as described in section 1.7.1.2, and maintained as described in section 3.3.3. For the elimination study, embryos were exposed to 10 mL of 10 µg/L ng/L ^{14}C-EE2 in a 6 well plate from 6 – 48 hpf. The amount of ^{14}C-EE2 per embryo was then assessed as described in section 3.3.4, at 0, 24, 48, 96, and 120 hours after returning to clean water (Table 11). Three replicates containing 60 embryos per replicate were completed. A non-linear regression and an ANOVA with a Tukey *post hoc* test was completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*).

Table 11: Exposure duration of Japanese medaka (*O. latipes*) to ^{14}C-EE2, Objective 2: Depuration study. The darkened line ▬ represents the time of exposure to 10 µg/L ^{14}C-EE2, and "Sampled" denotes the time point when the embryos were analyzed in hours post exposure.

	Hours Post Fertilization (hpf)			Hours Post Exposure					
Test #	6	24	48	0	24	48	72	96	120
1	▬	▬	▬	Sampled					
2	▬	▬	▬		Sampled				
3	▬	▬	▬			Sampled			
4	▬	▬	▬				Sampled		
5	▬	▬	▬					Sampled	
6	▬	▬	▬						Sampled

3.3.7. Objective 3: The Influence of HPβCD on the Uptake of EE2

Embryonic Japanese medaka were obtained as described in section 1.7.1.2, and maintained as described in section 3.3.3 At 120 hpf, 60 embryos per replicate were exposed to 10µg/L ^{14}C-EE2 alone, and to a mixture of 188 µg/L HPβCD and 10 µg/L ^{14}C-EE2. After 24 hours of exposure at 144 hpf, all treatments were assessed as described in section 3.3.4. A Student's T-test was conducted to compare the means to the two groups using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) at an alpha of 0.05.

3.4. Results

3.4.1. Objective 1: ^{14}C-EE2 Rangefinder Assay

In the rangefinder experiment, 40 medaka embryos were exposed to 100 ng/L, 1000 ng/L and 10000 ng/L ^{14}C-EE2 from 24 hpf to 120 hpf. Uptake was determined to be 112.0 ng, 415.0 ng, and 665.6 ng of ^{14}C-EE2 per embryo (Table S14). This data was used to justify a concentration of 10 µg/L ^{14}C-EE2 and 60 embryos per replicate for the following studies.

3.4.2. Objective 2: ^{14}C-EE2 Uptake and Elimination Assays

Embryonic uptake was assessed following a continual exposure to ^{14}C-EE2 for varying lengths of time. All exposures began at 6 hpf and were terminated at 24, 48, 96, and 168 hpf. A very strong positive sigmoidal correlation was observed and described by the equation $y=(1451.6818)/1+exp(-(x-85.1238)/23.7694))$ with an r^2 value of 0.9999 (Figure 11). Uptake in embryonic medaka appeared to be reaching a plateau at 168 hpf, with approximately 1400 ng of ^{14}C-EE2 per embryo. A significant difference in ^{14}C-EE2 uptake was observed between all exposure time points ($P \leq 0.05$).

The uptake of ^{14}C-EE2 over a 24 hour period was determined across embryogenesis. Exposures began at 6, 24, 48, 72, 96, 120, and 144 hpf, and sampling occurred 24 h after the initiation of exposure. A strong positive quadratic correlation was observed when comparing embryonic age that can be described by the equation $y=121.5052+(-0.9265)x+(0.0114)x$ with an r^2 value of 0.9350 (Figure 12). A notable point on the curve occurred when embryos were exposed at 6 hpf, where their mean uptake was

greater than the next age of exposure, 24 hpf, although not statistically significant (P>0.05). After this temporary decrease, the uptake of ^{14}C-EE2 continued on an upward trajectory (Figure 12). Uptake of ^{14}C-EE2 was significantly greater in embryos exposed from 144 to 168 hpf, compared to all other exposure windows ($P \leq 0.05$). Embryos exposed from 120 to 144 hpf also experienced significantly greater ^{14}C-EE2 uptake than 6 – 30, 24 – 48 and 48 – 72 hpf exposures ($P \leq 0.05$).

Elimination of ^{14}C-EE2 was assessed in Japanese medaka embryos every 24 hours following exposure from 6 – 48 hpf. At 0 hours post exposure, an average of 360 ng of ^{14}C-EE2 was taken up per embryo, and at 120 hours post exposure, this was reduced to an average of 165 ng of ^{14}C-EE2 per embryo. Elimination of ^{14}C-EE2 was characterized by a three parameter exponential decay equation (*y=139.5332+223.2979(exp(-0.0236(x)))* with an r^2 value of 0.9624 as a function of time (Figure 13). A significant decrease in the amount of ^{14}C-EE2 within each embryo was observed in all time points when compared to 0 hours post exposure ($P \leq 0.05$). Embryos from 48, 72, 96 and 120 hours post fertilization all contained significantly lower amounts of ^{14}C-EE2 when compared to 24 hours post exposure embryos. ($P \leq 0.05$). No significant differences were observed between 72, 96, and 120 hours post exposure embryos ($P > 0.05$) (Figure 13).

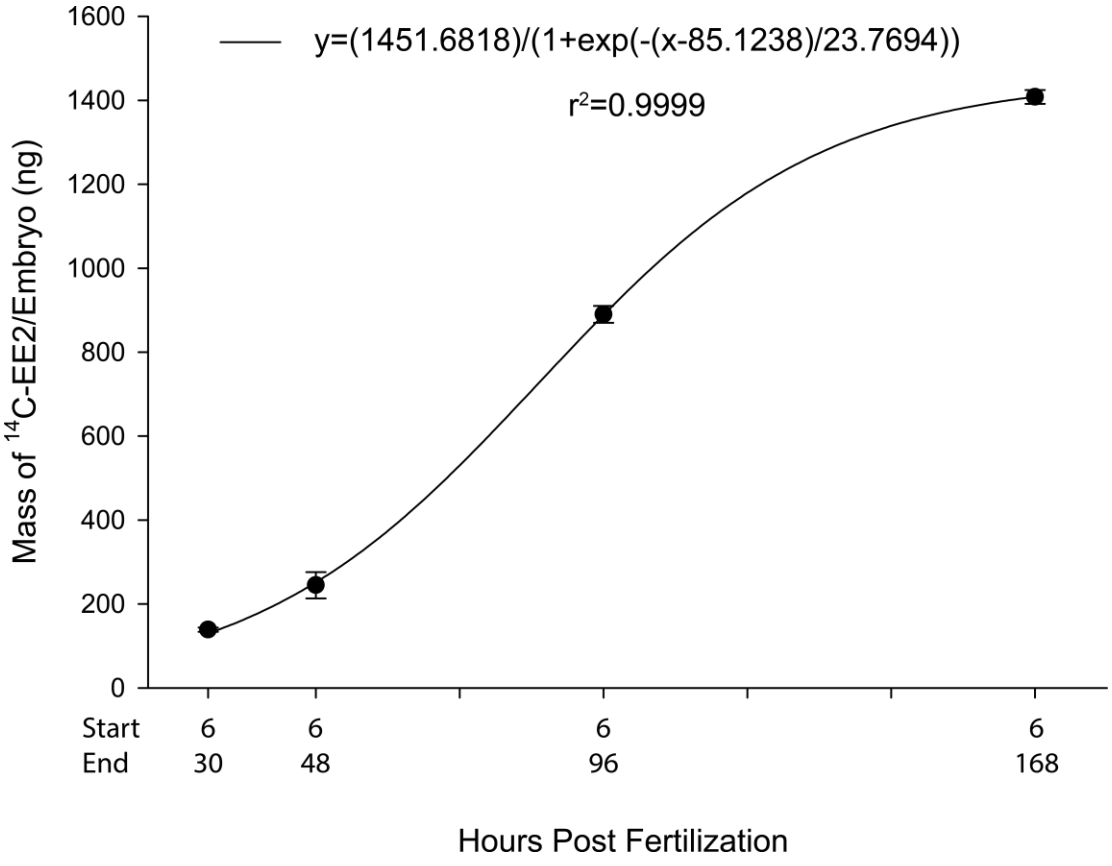


Figure 11: Mean mass ± standard error of ^{14}C-17α-ethinylestradiol (^{14}C-EE2) per Japanese medaka (*O. latipes*) embryo following a continuous exposure to 10 µg/L ^{14}C-EE2 6 – 30 hours post fertilization (hpf), 6 – 48 hpf, 6 – 96 hpf, and 6 – 168 hpf. All embryos were sampled at the end of the exposure window. Three replicates per time point with 60 embryos per replicate were tested. An equation of *y=(1451.6818)/1+exp(-(x-85.1238)/23.7694))* was generated with r^2=0.9999.

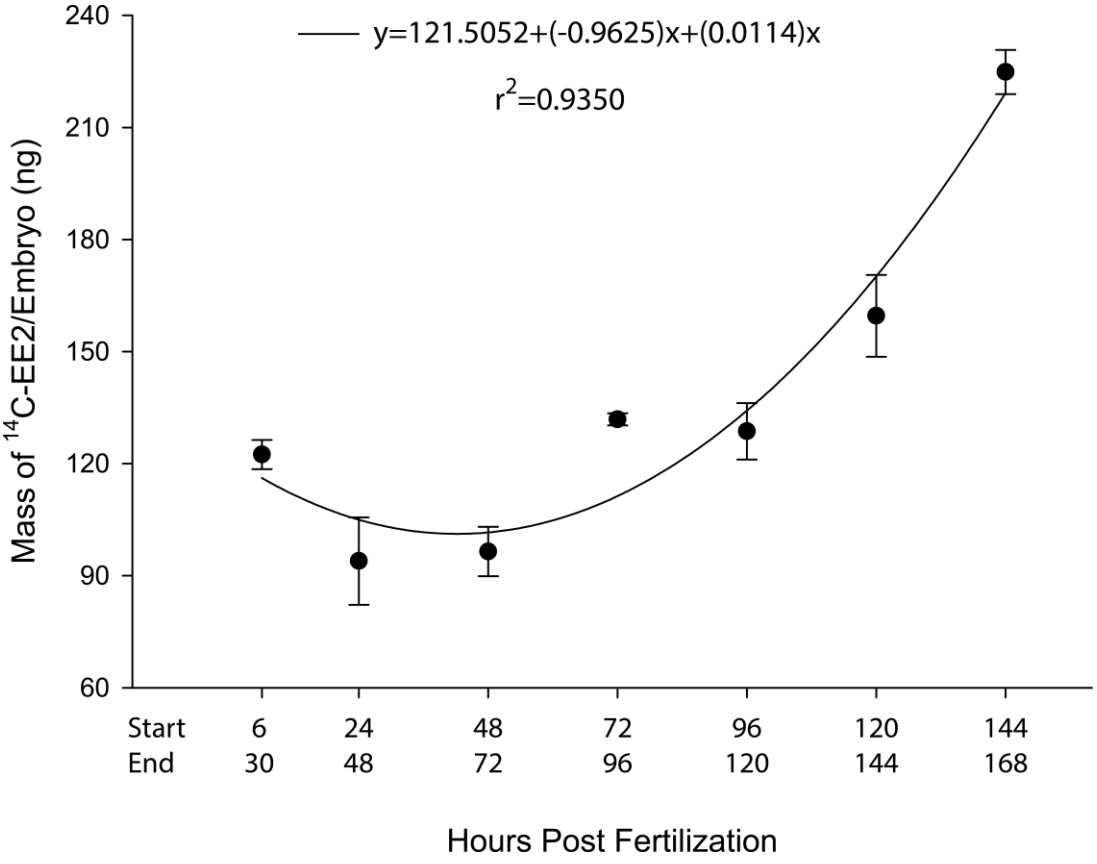


Figure 12: Mean mass ± standard error of ^{14}C-17α-ethinylestradiol (^{14}C-EE2) per Japanese medaka (*O. latipes*) embryo following a 24 hour pulse exposure to 10 µg/L ^{14}C-EE2 6 – 30 hours post fertilization (hpf), 24 – 48 hpf, 48 – 72 hpf, 72 – 96 hpf, 96 – 120 hpf, 120 – 144 hpf, and 144 – 168 hpf. All embryos were sampled at the end of the exposure window. Three replicates per time point with 60 embryos per replicate were tested. An equation of *y=121.5052+(-0.9265)x+(0.0114)x* was generated with r^2=0.9350.

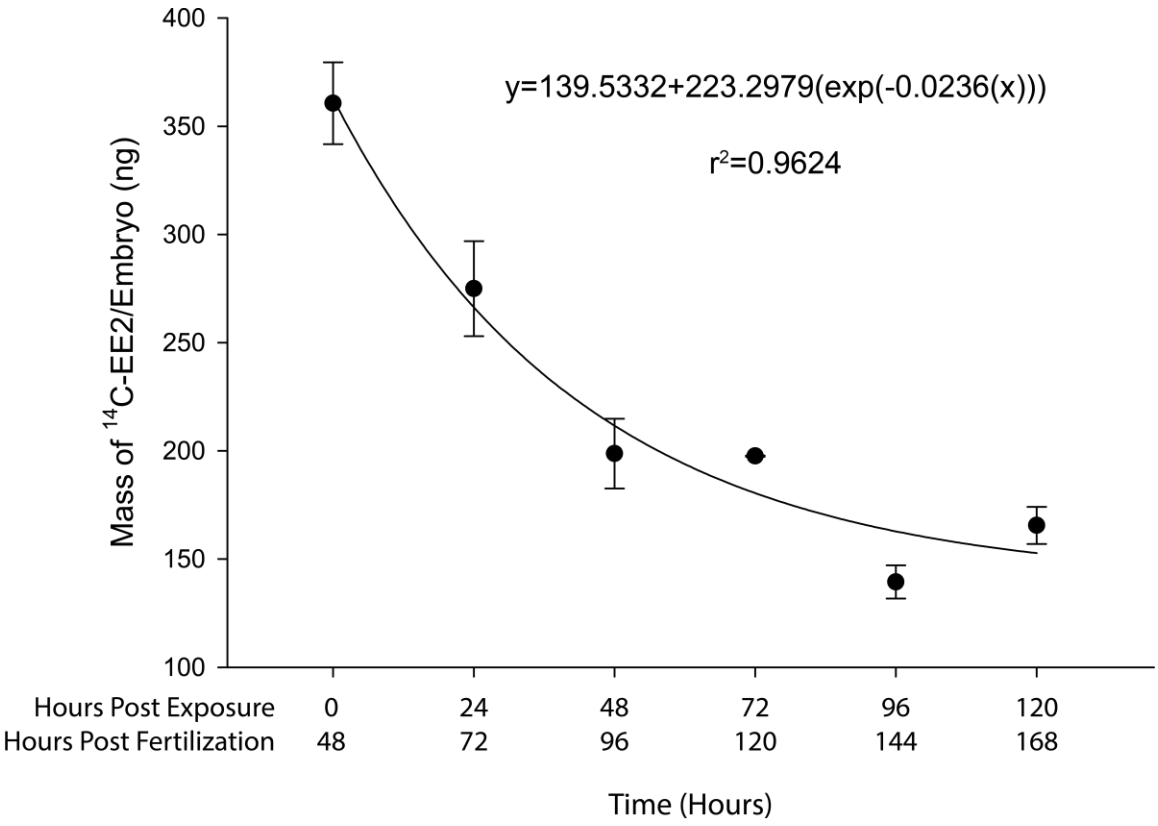


Figure 13: Mean mass ± standard error of ^{14}C-17α-ethinylestradiol (^{14}C-EE2) per Japanese medaka (*O. latipes*) embryo following an exposure to 10 µg/L ^{14}C-EE2 from 6 – 48 hours post fertilization (hpf). Following exposure embryos were placed in rearing solution and sampled at 0, 24, 48, 72, 96 and 120 hours post exposure. Embryo age, in hours post fertilization (hpf), is also indicated on the figure. Three replicates per time point with 60 embryos per replicate were tested. An equation of *y=139.5332+223.2979(exp(-0.0236(x)))* was generated with r^2=0.9624.

3.4.3. Objective 3: The Influence of HPβCD on the Uptake of ^{14}C-EE2

The role of HPβCD in the uptake of ^{14}C-EE2 into the chorion of 120 hpf embryonic Japanese medaka was assessed. The mass of ^{14}C-EE2 was determined following a 24 hour exposure to 10 µg/L ^{14}C-EE2 in the presence and absence of 188 µg/L HPβCD from 120 hpf to 144 hpf. The results demonstrated no significant difference in the mass of ^{14}C-EE2 within the chorion in ^{14}C-EE2 alone versus ^{14}C-EE2 + HPβCD treatments ($P > 0.05$) (Figure 14).

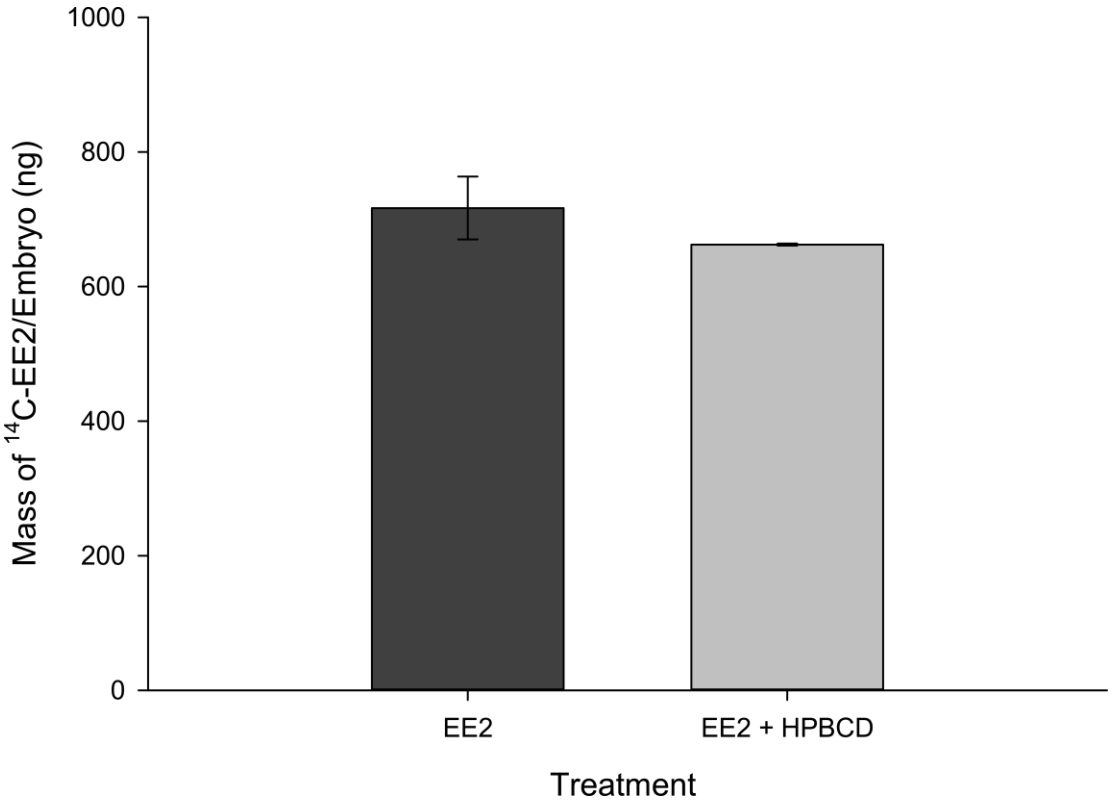


Figure 14: Mean mass ± standard error of ^{14}C-17α-ethinylestradiol (^{14}C-EE2) per Japanese medaka (*O. latipes*) embryo following a 24 hour pulse exposure to 10 µg/L ^{14}C-EE2 and 10 µg/L ^{14}C-EE2 + 188 µg/L (1:4 molar ratio) hydroxypropyl-β-cyclodextrin (HPβCD) from 120 – 144 hours post fertilization (hpf). All embryos were sampled at the end of the exposure window. Three replicates per time point with 60 embryos per replicate were tested. Significant difference from EE2 alone is represented by (*) ($P \leq 0.05$), Students t-test.

3.5. Discussion

A common assumption when assessing toxicity is a toxicants ability to interact, in some way, with a target site. This can be particularly challenging when studying embryo toxicity, as the chorion is an acellular envelope that separates developing embryos from the surrounding environment and thus can act as an additional biological barrier(Hart, 1983). In addition to chorion permeability, toxicity assessment during embryogenesis can be uniquely challenging due to the rapid developmental changes that occur in this phase. Toxicity is often transient or delayed depending on organ development, metabolism, and the presence of target sites. Currently, few studies have examined the uptake and elimination of PPCPs in embryonic fish. In this chapter, we examined the uptake and elimination of 17α-ethinylestradiol (EE2) across Japanese medaka (*Orizias latipes*) chorions at various stages of embryonic development. This was of particular interest given the observed transient effect of EE2 on embryonic heart rate observed in Chapter 2. It was also observed in Chapter 2 that hydroxypropyl-β-cyclodextrin (HPβCD) impeded this toxic effect of EE2. It was hypothesized that uptake of EE2 may have been reduced due to inclusion into the relatively large HPβCD molecule, ~1.4 kDa. The general aims of this chapter was to further understand the uptake and elimination of EE2 at various stages of development, and in the presence and absence of HPβCD from 120 – 144 hpf.

The first study in this chapter examined the uptake at following continuous exposure to radiolabeled 10µg/L EE2 (^{14}C-EE2) at various stages of embryonic development. It was observed that as the length of exposure increased across embryogenesis, the amount of ^{14}C-EE2 increased in a sigmoidal fashion (Figure 11). Maximal uptake was approached at 168 hpf with approximately 1400 ng of ^{14}C-EE2 per

embryo; further assessment was not possible due to hatching beyond this time point. This demonstrated that ^{14}C-EE2 is able to cross the chorion of embryonic medaka. Furthermore, it showed that uptake of ^{14}C-EE2 increases to a plateau when nearing hatch. Next, uptake was examined during 24 hour exposure windows across embryogenesis. A similar response was observed, with uptake increasing exponentially as embryogenesis progressed (Figure 12), indicating that permeability also increases dramatically as development progresses. This is supported by the observation that the chorion of fish becomes more permeable as embryogenesis progresses due to increasing size of chorion pore canals, and the breakdown of the chorion at stage 34, 120 hpf (Rubstov, 1981; González-Doncel *et al.*, 2004; Adams *et al.*, 2005).

Uptake in this manner was expected since EE2 is a small lipophilic compound. This result is supported by observations of estradiol (E2) uptake in zebrafish (*Danio rerio*), where chorion permeability increased with the age of the embryo (Souder and Gorelick, 2017). Additionally, little difference in uptake was observed in intact embryos versus intact embryo exposure followed by manual chorion removal (Souder and Gorelick, 2017), providing additional evidence that E2 is taken up across the chorion and into the developing embryo. Given the chemical similarities, it is expected that EE2 would behave in the same way as E2 (Table 2). In another study exposing embryonic medaka to radiolabeled EE2, EE2 was also taken up across the chorion (Bhandari *et al.*, 2015). When compared to the findings in Chapter 2, this result is consistent with a greater response as time progressed, until approximately 144 -168 hpf. These results became somewhat contradictory as hatching approached, indicating that another mechanism such as detoxification could have been initiated at this time point (Figure 3 and Figure 4).

The third study in this chapter looked at elimination of EE2 in embryonic medaka in an attempt to elucidate the cause of the observed transient toxicity of EE2. Given that the absolute amount of ^{14}C-EE2 continued to increase with time during exposure, elimination was not a leading hypothesis. Nonetheless, we felt it was important to investigate this endpoint to gain a better understanding of EE2 interaction with the embryo. It was observed that elimination of EE2 occurred in an exponential decay manner (Figure 13). As time progressed, the rate of EE2 elimination slowed, indicating that after 120 hours of depuration at 168 hpf, some amount of EE2 remained within the embryo. This was expected given the lipophilic nature of EE2. It has also been reported that E2 can be taken up by the yolk, indicating a potential location for EE2 sequestration (Souder and Gorelick, 2017).

Although uptake and elimination of ^{14}C-EE2 did not provide plausible evidence for the observed transient effect, detoxification may still be occurring. A major limitation of using radiolabeled isotopes in toxicological work is that it only reports the absolute amount of ^{14}C present in a sample. Therefore, it does not take into consideration metabolism of the parent drug into inactive or metabolized forms. This is a potential area of future research, which would aid in explaining the observed transient effects in this study as well as defining when metabolism becomes active in embryonic fish.

The last portion of this chapter looked into the effects of HPβCD on EE2 uptake. As described in Chapter 2, HPβCD appears to reduce the effect of EE2 on embryonic heart rates with the exception of exposure at 72 and 96 hpf. A potential explanation of this result was that HPβCD reduces EE2 uptake across the chorion. However, our results showed no change in EE2 uptake across the chorion during a 24 hour exposure from 120 – 144 hpf

(Figure 14). These results were somewhat expected given the size of HPβCD, ~1.4 kDa, and the diameter of chorion pore canals, 3-4 kDa. Although these results did not support our hypothesis, uptake into the developing embryo and within the cells could still be hindered by HPβCD. Future research into uptake beyond the chorion should be investigated since HPβCD is thought to have difficulty crossing cellular membranes due to size and hydrophilicity limiting the ability for EE2 to interact with GPER (Oishi *et al.*, 2008). Exposing intact embryos to ^{14}C-EE2 + HPβCD, followed by manual chorion removal and then quantifying ^{14}C-EE2 uptake within the embryonic fish could potentially address this question. Furthermore, assessment of ^{14}C-EE2 uptake in the presence of HPβCD at various time points should be determined due to the reported initiation of chorion breakdown at 120 hpf in Japanese medaka (González-Doncel *et al.*, 2004).

In summary, this chapter identified that EE2 was readily taken up by embryonic Japanese medaka, further supporting the susceptibility of fish embryos to EE2. Although identification of how HPβCD impedes EE2 toxicity was not determined, future research avenues have been presented and should be investigated.

Chapter 4: The impact of EE2 on Embryonic Oxygen Consumption (MO_2)

4. Chapter 4: The impact of EE2 on Embryonic Oxygen Consumption (MO_2)

4.1. Introduction

The use of apical endpoints such as growth, reproduction, and metabolism aid in the understanding of how a particular toxicant may impact the overall fitness of an organism. When trying to relate laboratory findings to more relevant higher levels of biological organization such as population effects, these apical endpoints are critical. When using sub-apical endpoints such as heart rate or gene transcription, it is thus advantageous to relate these observed effects with corresponding apical endpoints. Although sub-apical endpoints often help elucidate a mode of action or identify a molecular initiating event, the linkage to an apical endpoint is not always clear (Miracle and Ankley, 2005). Understanding this is very important when conducting ecological risk assessments (Miracle and Ankley, 2005; Ankley *et al.*, 2010). Thus, the effects of EE2 on embryonic metabolic rate was examined in this chapter to see if there was a link to the observed EE2 induced effects on heart rate (Chapter 2). The benefits of assessing heart rate rather than MO_2, is due to ease of measurement, lower associated costs, and minimal specialized equipment. However, MO_2 is a more direct measure of metabolism, and is commonly measured using a swim tunnel or microplate respirometers. Alterations in MO_2 can limit the fitness of a fish within an ecosystem. For example, decreases in MO_2 have been known to slow growth, and impede the energetics of an organism (Houde and Schekter, 1983). Since most fish are ectotherms, water temperature is an important modifying factor of metabolic rate, and thus must be controlled for (Vornanen, 2016).

The Fick principle describes the relationship between metabolic rate and heart rate, in terms of oxygen consumption, (Equation 6) (Priede and Tytler, 1977). This principle has been applied with success to birds and mammals, however, its relation to fish appears to be less direct. It was demonstrated in rainbow trout (*Oncorhynchus mykiss*) that changes in oxygen demands were achieved by adjusting stroke volume with minor changes in heart rate (Stevens and Randall, 1967). This suggested that heart rate may not be the best measure of metabolism in fish. However, a study by Folkerts *et al.*, (2017), demonstrated that hydraulic fracturing flow-back and produce water caused a decrease in larval zebrafish heart rate. They supported this finding by using a Loligo® Microplate respirometer which showed a decrease in MO_2.

$$MR = HR \times Stroke\ Volume \times (A - V\ difference)$$

Equation 6: The Fick principle, where MR represents metabolic rate, HR represents heart rate, the stroke volume is the volume of one heart beat, and the A – V difference is the difference in oxygen content of arterial and venous blood (Priede and Tytler, 1977).

A major goal of this chapter was to determine if there is a link between the EE2 induced heart rate reduction and MO_2 in embryonic Japanese medaka (*Oryzias latipes*). As with Chapters 2 and 3, we wanted to also determine if hydroxypropyl-β-cyclodextrin (HPβCD) had the ability to alter any potential EE2 induced MO_2 changes. We hypothesized

that HPβCD will lessen the effects of EE2 given that we have previously demonstrated similar effects of HPβCD co-exposure on heart rates (Chapter 2).

4.2. Objectives, Knowledge Gaps, Rationale, and Hypotheses

4.2.1. Objective 1: EE2 and Embryonic MO_2

The first objective of this chapter was to provide physiological relevancy to the alterations in embryonic heart rate that was observed in Chapter 2. We thus investigated if EE2 exposure altered the MO_2 of embryonic Japanese medaka. To the best of our knowledge, no research has been previously published on the impacts of EE2 on the MO_2 of embryonic fish.

The null hypothesis for this experiment was:

H_0: There are no differences in the oxygen consumption rate (MO_2) between embryonic Japanese medaka exposed to EE2 and the carrier control (0.01 % v/v acetone).

4.2.2. Objective 2: Impact of EE2 and HPβCD on Embryonic MO_2

The second objective was to determine if the effects of EE2 on embryonic MO_2 could be altered by the presence of HPβCD. In Chapter 2, it was demonstrated that HPβCD mitigated the effects of EE2. The aim in this chapter was to identify if this reduction in toxicity could be observed on MO_2, providing direct physiological relevancy.

The null hypothesis for this experiment was:

H_0: There are no differences in the oxygen consumption rate (MO_2) between embryonic Japanese medaka exposed to EE2 and EE2 + HPβCD.

4.3. Materials and Methods

4.3.1. Objective 1: EE2 and Embryonic MO_2

4.3.1.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), was dissolved in acetone. EE2 treatments were made through serial dilutions with rearing solution as the diluent, providing for final nominal exposure concentrations of 0 ng/L (carrier control), 1 ng/L, 10 ng/L, 100 ng/L, 1000 ng/L, and 10 000 ng/L EE2. All treatments had an acetone concentration of 0.01 % v/v.

4.3.1.2. Experimental Procedure

Embryonic Japanese medaka were obtained and maintained as described in section 1.7.1.2. At 24 hpf, 5 embryos per treatment were exposed in a 24 well plate to a carrier control (0.01 % v/v acetone), 1 ng/L, 10 ng/L, 100 ng/L, 1000 ng/L, and 10 000 ng/L EE2, with 2 ml of treatment solution per well with 6 replicates per treatment. All solutions were renewed daily, and at 48 hpf, embryos were thinned to 3 embryos per replicate (Table 12). Exposure and MO_2 measurements were conducted in a 27° C temperature control room. Embryonic oxygen consumption rate (MO_2) was monitored at 120 and 144 hpf with a 24 well Loligo® Systems Microplate respirometer (*Viborg, Denmark*). During measurement, three embryos from each replicate were transferred from the 24 well exposure plate into

the wells of the respirometer, with 6 blank wells filled with control solution and containing no embryos. The wells were filled to the top with the corresponding treatment solution, minimizing headspace. The respirometer was sealed with Parafilm from Bemis North America (*Neenah, Wisconsin, USA*), a weighted block supplied by Loligo® Systems (*Viborg, Denmark*) was placed on top to aid in sealing. The plate was then placed on the reader. Readings occurred on a plate shaker set to 60 rpm and MO_2 was monitored for 30 minutes. After the reading was complete, the embryos were returned to their original 24 well exposure plate. The same embryos were sampled at 120 and 144 hpf.

Data was analyzed by calculating mean oxygen consumption (pmol/min) for each treatment group. After testing for normality and homogeneity, an ANOVA with a Dunnet's *post hoc* test was used to determine a difference between treatments and the control. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) and run at an alpha of 0.05.

Table 12: Exposure summary for EE2 impact on metabolic rate of embryonic Japanese medaka (*O. latipes*). Nominal exposure concentrations of 17α-ethinylestradiol (EE2), number of replicates, and number of embryos per replicate are listed.

Treatment #	[EE2] (ng/L)	Acetone (%)	Number of replicates	Number of embryos per replicate
1	0	0.01	6	3
2	1	0.01	6	3
3	10	0.01	6	3
4	100	0.01	6	3
5	1000	0.01	6	3
6	10000	0.01	6	3

4.3.2. Objective 2: Impact of EE2 and HPβCD on Embryonic MO_2

4.3.2.1. Chemicals

Rearing solution was made in house and comprised of 10 % NaCl, 0.30 % KCl, 0.40 % CaCl2·2H2O, 1.63 % MgSO4·7H2O, 0.01 % methylene blue, and distilled water. EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3, 17-diol), purchased from Sigma-Aldrich (*Oakville, Ontario, Canada*), was dissolved in acetone, and Hydroxypropyl-β-cyclodextrin (HPβCD), 97.0 %, was purchased from Acros Oganics (*New Jersey, USA*) was dissolved in deionized water. Treatment solutions were made using rearing solution as a diluent. Nominal treatments consisted of a carrier control, 10 ng/L EE2, 188 ng/L HPβCD, 10 ng/L EE2 + 47 ng/L HPβCD, 10 ng/L EE2 + 94 ng/L HPβCD, and 10 ng/L EE2 + 188 ng/L HPβCD. An acetone concentration of 0.01 % v/v was maintained in each treatment.

4.4. Experimental Procedure

Embryonic Japanese medaka were obtained and maintained as described in section 1.7.1.2. At 24 hpf, 5 embryos per treatment were exposed in a 24 well plate to a carrier control (0.01 % v/v acetone), 10 ng/L EE2, 188 ng/L HPβCD, 10 ng/L EE2 + 47 ng/L HPβCD, 10 ng/L EE2 + 94 ng/L HPβCD, and 10 ng/L EE2 + 188 ng/L HPβCD, with 2 ml of treatment solution per well with 6 replicates per treatment. All solutions were renewed daily and at 48 hpf embryos were thinned to 3 embryos per treatment (Table 13). Exposure and MO_2 measurements were conducted in a 27° C temperature control room.

Embryonic oxygen consumption rate (MO_2) was monitored at 120 and 144 hpf with a 24 well Loligo® Systems Microplate respirometer (*Viborg, Denmark*). During oxygen consumption measurement, three embryos from each treatment were transferred from the 24 well exposure plate into the wells of the respirometer, with 6 blank wells filled with control solution and containing no embryos. The wells were filled to the top with the corresponding treatment solution, minimizing headspace. The respirometer was sealed with Parafilm from Bemis North America (*Neenah, Wisconsin, USA*), a weighted block supplied by Loligo® Systems (*Viborg, Denmark*) was placed on top to aid in sealing. The plate was then placed on the reader. Readings occurred on a plate shaker set to 60 rpm as per the manufacturer's instructions. MO_2 was monitored for 30 minutes. After the reading was complete, the embryos were returned to their original 24 well exposure plate.

Data were analyzed by calculating mean oxygen consumption (pmol/min) for each treatment group. After testing for normality and homogeneity, an ANOVA with a Tukey post hoc test was used to determine a difference between treatments. All statistics were completed using Sigmaplot 13.0 (*Statsoft, Inc. San Jose, USA*) and run at an alpha of 0.05.

Table 13: Exposure summary for EE2 impact on metabolic rate of embryonic Japanese medaka (*O. latipes*). Nominal concentrations of 17α-ethinylestradiol (EE2) and hydroxypropyl-β-cyclodextrin (HPβCD), number of replicates, and number of embryos per replicate are listed.

Treatment #	[EE2] (ng/L)	HPβCD (ng/L)	Acetone (%)	Number of replicates	Number of embryos per replicate
1	0	0	0.01	6	3
2	10	0	0.01	6	3
3	0	188	0.01	6	3
4	10	47	0.01	6	3
5	10	94	0.01	6	3
6	10	188	0.01	6	3

4.5. Results

4.5.1. Objective 1: EE2 and Embryonic MO_2

Oxygen consumption (MO_2) of embryonic Japanese medaka was assessed at 120 and 144 hpf following exposure to a range of EE2 concentrations. At 120 hpf, a mean MO_2 of 74.7 pmol/min was observed (Figure 15). This was significantly greater than embryos exposed to 1 ng/L EE2, which had a mean of 47.2 pmol/min ($P \leq 0.05$) (Figure 15). Exposure to 10, 100, 1000, and 10000 ng/L EE2 resulted in no significant change from control ($P > 0.05$).

At 144 hpf, the MO_2 of control embryos was 81.6 pmol/min (Figure 16). No significant differences in MO_2 were observed in any of the EE2 exposures when compared to the control ($P > 0.05$). However, a trend of increasing MO_2 with an increasing EE2 concentration was observed, with the lowest MO_2, 56.97 pmol/min occurring at 1 ng/L EE2 and the highest being 103.49 pmol/min at 1000 ng/L EE2 (Figure 16).

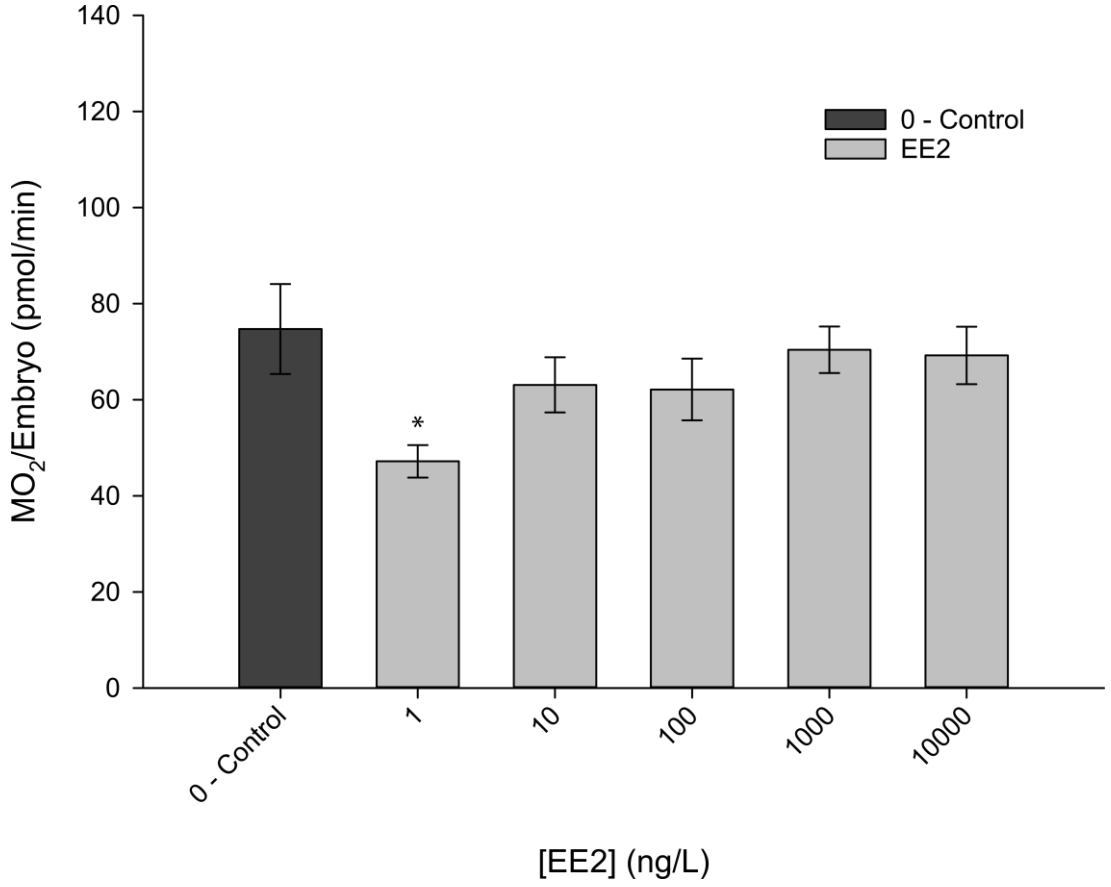


Figure 15: Mean oxygen consumption (MO_2) ± standard error per embryo of 120 hour post fertilization (hpf) Japanese medaka (*O. latipes*) exposed to 0 (carrier control, 0.01 % v/v acetone) 1, 10, 100, 1000, and 10000 ng/L 17α-ethinylestradiol (EE2). Each treatment consists of six replicates containing 3 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA, Tukey *post hoc*.

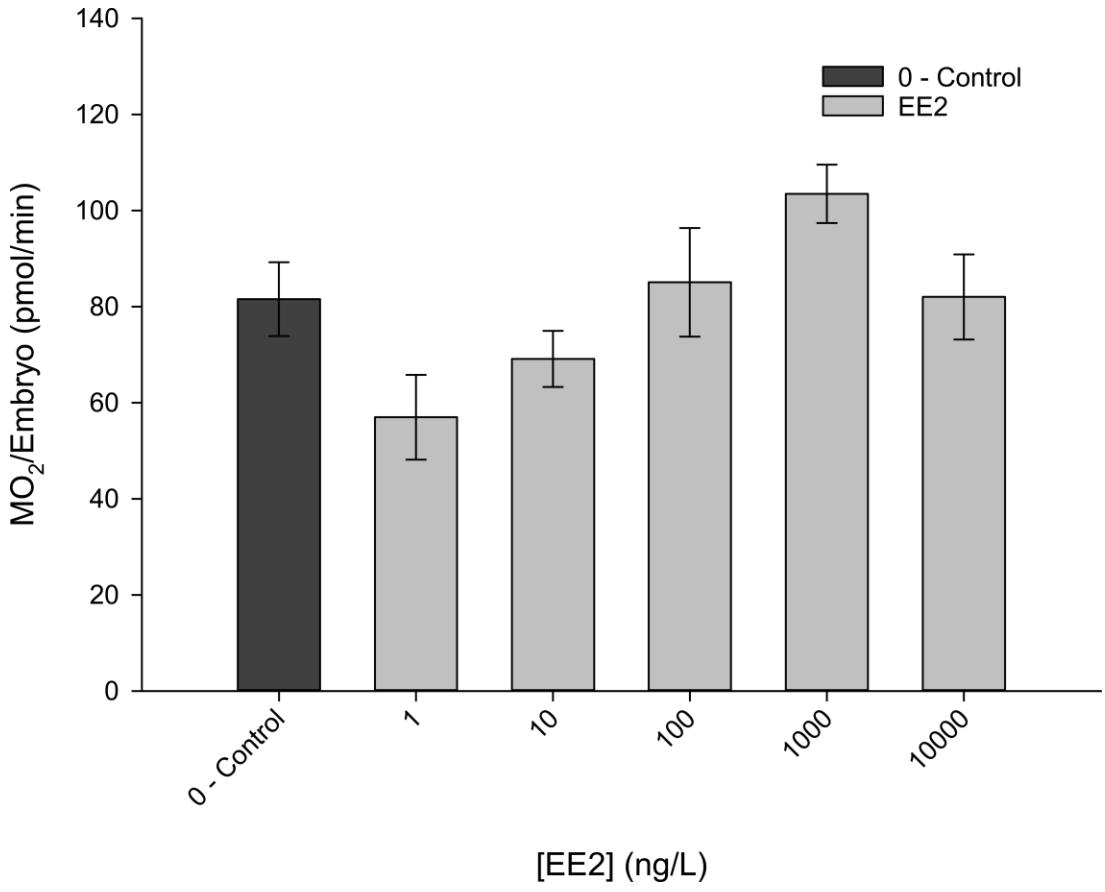


Figure 16: Mean oxygen consumption (MO_2) ± standard error per embryo of 144 hour post fertilization (hpf) Japanese medaka (*O. latipes*) exposed to 0 (carrier control, 0.01 % v/v acetone) 1, 10, 100, 1000, and 10000 ng/L 17α-ethinylestradiol (EE2). Each treatment consists of six replicates containing 3 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.0.5$), ANOVA.

4.5.2. Objective 2: Impact of EE2 and HPβCD on Embryonic MO_2

The mean MO_2 of embryonic Japanese medaka was assessed at 120 and 144 hpf following an exposure to EE2 alone, HPβCD alone, and a combination of EE2 + HPβCD. At 120 hpf, a mean MO_2 of 55.85 pmol/min was observed in the control embryos. Embryos exposed to 10 ng/L EE2, and 188 ng/L HPβCD alone, resulted in an MO_2 of 49.05 pmol/min and 65.87 pmol/min, respectively, neither of which was significantly different from the control ($P > 0.05$) (Figure 17). Likewise, embryos exposed to EE2 and HPβCD simultaneously, were not significantly different from the control ($P > 0.05$) (Figure 17).

Mean MO_2 at 144 hpf showed a similar result as 120 hpf, with no significant differences between the treatments and the controls ($P > 0.05$). Control embryos experienced a mean MO_2 of 48.39 pmol/min, while embryos exposed to EE2 and HPβCD alone had a mean MO_2 of 51.33 and 51.39 pmol/min respectively (Figure 18). Embryos exposed to EE2 in combination with HPβCD had a mean MO_2 between 50.80 pmol/min and 59.10 pmol/min, with no apparent trend (Figure 18).

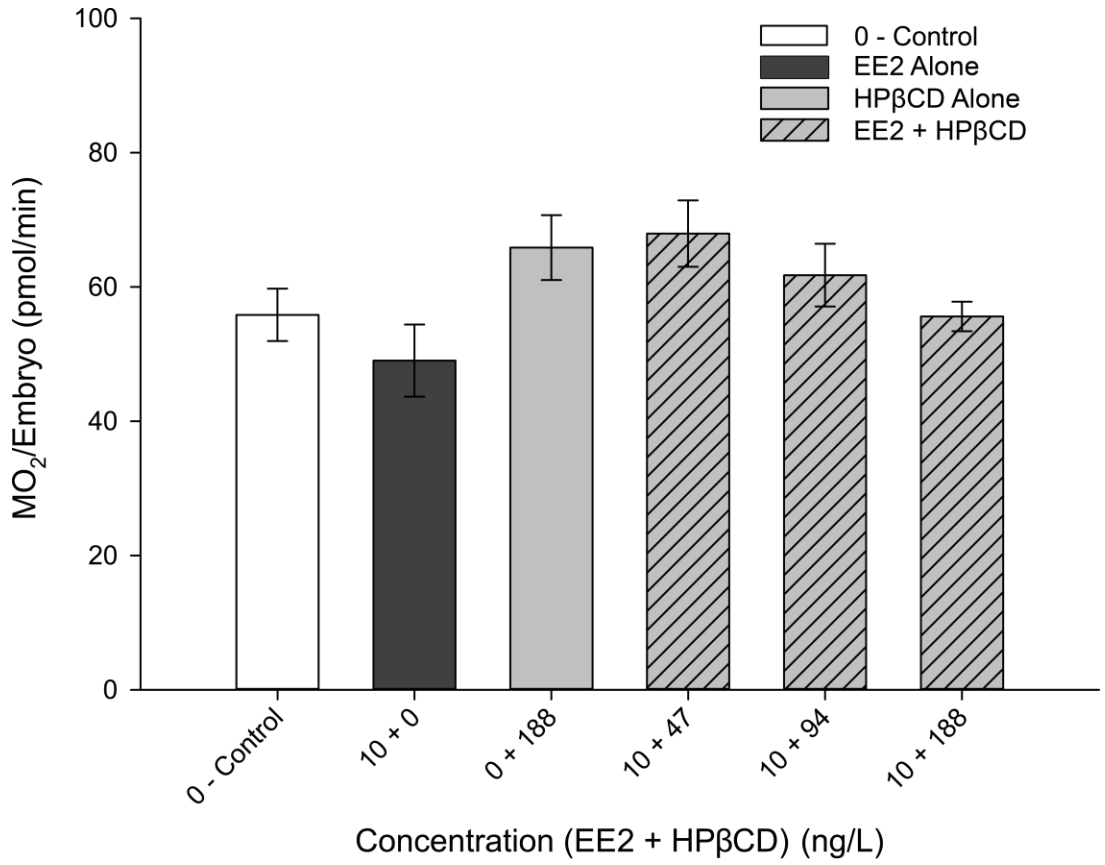


Figure 17: Mean oxygen consumption (MO_2) ± standard error per embryo of 120 hour post fertilization (hpf) Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, hydroxypropyl-β-cyclodextrin (HPβCD) alone, or EE2 + HPβCD. Each treatment consists of six replicates containing 3 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA.

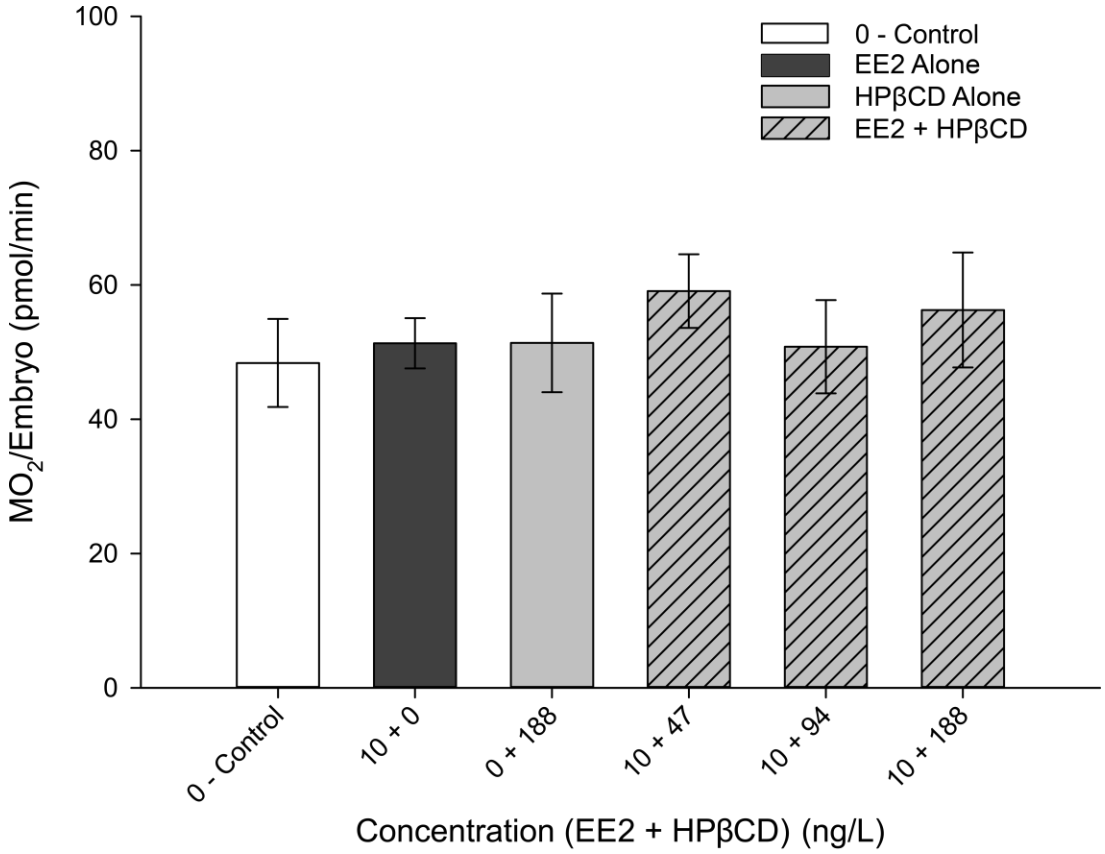


Figure 18: Mean oxygen consumption (MO2) ± standard error per embryo of 144 hour post fertilization (hpf) Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, hydroxypropyl-β-cyclodextrin (HPβCD) alone, or EE2 + HPβCD. Each treatment consists of six replicates containing 3 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA.

4.6. Discussion

Balance between energy input and output is critical to all organisms, including fish. In fish, many apical endpoints such as growth and survival can be impacted by an imbalance of energy (Houde and Schekter, 1983). These effects are often amplified during vulnerable life-stages. For example, spawning fish may experience limited food availability and high energy expenditure; embryonic fish have a finite energy supply stored as yolk. Perturbations in metabolic rate during sensitive life-stages, including embryogenesis may have significant impacts on fish development and growth (Woltering, 1984; Koger *et al.*, 2000). Energy consumption is commonly measured through oxygen consumption or heart rate.

Recently, there has been a strong push in the field of aquatic toxicology to develop adverse outcome pathways (AOPs) (Ankley *et al.*, 2010) In brief, AOPs are a method of linking molecular imitating events with associated biological responses. The goal of AOPs are to eventually aid in the prediction of toxic responses to a chemical. While the scope of this thesis was not to develop an alternative AOP of 17α-ethinylestradiol (EE2), the idea of linking EE2 activation of GPER to alterations in heart rate and oxygen consumption (MO_2) was of interest. The general aim of this chapter was to determine if the observed changes in heart rate in Chapter 2 could be detected as changes in MO_2.

In this chapter, decreased MO_2 was observed in 120 hpf Japanese medaka (*Oryzias* latipes) embryos exposed to 1 ng/L of EE2 (Figure 15). This datum point was the only correlated with the decrease in heart rate observed in Chapter 2. A trend of increasing MO_2 with increased EE2 concentration was detected at 144 hpf. Unlike the observations of

Folkerts *et al.*, (2017), out results did not closely relate heart rate to MO_2. Furthermore, MO2 was also not changed in the presence of EE2 and HPβCD.

There is evidence that suggests that heart rate is not a reliable measure of metabolism in fish. In rainbow trout, alteration in stroke volume rather than heart rate is used to meet changing oxygen demands (Equation 6) (Stevens and Randall, 1967). Our data supports this argument and could explain the lack of relationship between the two endpoints. Additionally, embryonic teleost fish rely heavily on cutaneous gas exchange to meet metabolic demands (Pelster and Burggren, 1996; Wells and Pinder, 1996; Rombough and Ure, 2017). Since cutaneous gas exchange does not rely on the circulatory system for transport, cardiac output may also not be linked closely to metabolism at this life-stage. However, it has been demonstrated that metabolic demands and cardiac output do become more closely linked as development progresses (Pelster and Burggren, 1996). It would be valuable to determine if larval medaka express a tighter relationship between heart rate and metabolic rate upon exposure to EE2.

The results presented in this chapter and supporting literature have indicated that changes in the heart rate of fish embryos do not tightly correlate to metabolism. However, it should be noted that estradiol (E2) has been linked to metabolism changes through insulin regulation in mammalian models (Sharma and Prossnitz, 2011). Insulin is a peptide hormone secreted by beta cells of the pancreas, and is responsible for glucose homeostasis. When blood glucose is high, insulin promotes the absorption of circulating glucose from the blood into the liver and fat where it is converted to glycogen or fats (Sonksen and Sonksen, 2000). During times of low blood sugar, insulin is down regulated and glucagon

is secreted by alpha cells. Glucagon stimulates the liver to release glucose through gluconeogenesis, effectively opposing the effects of insulin (Sonksen and Sonksen, 2000).

In ovariectomized mice, which exhibited low levels of E2, the promotion of subcutaneous fat and inhibition of visceral fat was observed. Moreover, in GPER knockout mice, increased blood glucose, decreased insulin release, lower energy expenditure and increased weight have been observed; this indicates that may GPER have a role in metabolism via insulin secretion (Haas *et al.*, 2009; Sharma *et al.*, 2013; Davis *et al.*, 2014). It is also speculated that glucose intolerance could be the result of elevated circulation of lipids and insulin resistance (Sharma *et al.*, 2018). Furthermore, GPER knockout male mice were found to have an abnormal lipid profile with elevated levels of pro-inflammatory cytokines, and a decrease in the adipose-specific cytokine adiponectin (Sharma *et al.*, 2013). This highlights that GPER may have a role in regulating inflammation and the lipid profile of mice.

EE2 has also been shown to decrease growth in fish. Zebrafish exposed to 25 ng/L EE2 were significantly shorter in length and lighter in body weight compared to the control (VANDENBELT *et al.*, 2003). These growth effects are consistent with the interaction of E2 and GPER described in mice.

Insulin in fish has been primarily studied in salmonid fish, and has been reported to be structurally similar to other vertebrate insulins (Navarro *et al.*, 2014). However, a major difference between fish and mammalian insulin secretion response does exist, particularly in carnivorous fish. In fish, insulin secretion tends to respond more strongly to amino acids than glucose and varies between species (Navarro *et al.*, 2014). This suggests that GPER may have a different metabolic role in fish than in mammals. The non-genomic role of

GPER in the metabolic effects of fish thus presents an interesting area of research and should be further investigated.

While data from this chapter did not clearly demonstrate significant metabolic effects of EE2, there remains evidence that GPER plays an important role in metabolism through insulin regulation in mice. The lack of conclusive metabolic effects could be due to a lack of pancreatic development and function during the life stage tested, or biological differences between mice and Japanese medaka. Additionally, the subtlety of change may require a greater length of observation in order to tease out a metabolic effect. Given the known growth effects of EE2 and our increased understanding of GPER function, future research into the metabolic effects of EE2 in fish should be pursued.

Chapter 5: Overall Conclusions

5. Chapter 5: Overall Conclusions

The overall goal of this thesis was to identify a non-genomic mode of action for 17αethinylestradiol (EE2) toxicity and the influence of hydroxypropyl-β-cyclodextrin (HPβCD). We approached this project from an environmental perspective, prioritizing low, environmentally relevant concentrations of EE2. In this study, reduced embryonic heart rate of Japanese medaka (*Oryzias latipes*) was the most prominent biomarker of EE2 exposure. Supported by previous literature, it was demonstrated that the G protein-coupled estrogen receptor (GPER) was responsible for these changes in heart rate. This research is the first to demonstrate that GPER activation can reduce the embryonic heart rate of Japanese medaka at low concentrations. As GPER research has grown, it has become apparent that it may be responsible for many of the non-genomic effects of estrogen signaling.

Use of embryonic Japanese medaka allowed for relatively high throughput study of these endpoints. Medaka exhibit high reproductive output and a clear chorion allows for clear observation of their hearts. Embryonic medaka blood is a vibrant red and very apparent during embryonic development. The length of medaka embryogenesis typically lasts 10 days, allowing for greater time of embryonic observation than in many other species of fish. For comparative purposes, embryogenesis of zebrafish (*Danio rerio*), another commonly studied fish for embryonic development and toxicity studies, typically lasts only four days (Romano *et al.*, 2017). Depending on the endpoints investigated, developmental time can be an important factor in understanding toxicity (Bhandari *et al.*, 2015). In this thesis, the greater length of time was beneficial for the determination of

exposure effects as organogenesis progressed. However, variability was high at early and later stages of development highlighting an area of future research.

Embryonic heart rate was investigated as the primary endpoint (biomarker) of chemical exposure in this thesis. Cardiac function, the driving force of the circulatory system, is responsible for delivering nutrients, removing waste, and gas exchange. The heart rate of vertebrates can be controlled by internal signaling as well as exposure to toxicants. We observed a decrease in heart rate following exposure to nominal concentrations as low as 0.1 ng/L EE2. We also determined through specific estrogen receptor agonists and antagonists that GPER was responsible for this effect. These results indicate that embryonic heart rate may be a good indicator of GPER activation as it is not influenced by ERα or ERβ activation. A knowledge gap that still remains following this research is an understanding of the downstream signaling of GPER at environmentally relevant concentrations of EE2. Given the many reported roles of GPER, determining this downstream signaling in fish is of value. Information from this future research may further elucidate the non-genomic responses of fish to EE2 exposure.

The effects observed in Chapter 2 appeared to be transient in nature, with the heart rate of exposed embryos returning to control levels as well as increased variability as hatching approached. This led us to investigate the uptake of EE2 across the chorion. Uptake followed a sigmoidal pattern with permeability increasing with embryonic age. As expected, elimination followed an exponential decay pattern, but did not provide plausible evidence for the observed transient effect on heart rate. A major limitation of using radiolabeled isotopes in toxicological work is that only the absolute amount of ^{14}C present in a sample is determined. This method does not take into consideration the potential

metabolism of the parent drug into inactive or metabolized forms. Future research into this aspect may help to explain the transient effects observed in this study and could further define when metabolism of EE2 becomes active in embryonic fish.

Another goal of this thesis was to determine if a link existed between the observed effects on heart rate and changes in metabolic rate as measured by embryonic oxygen consumption. It was concluded that heart rate was not a good predictor of oxygen consumption in embryonic fish. This is supported by the fact that embryonic fish also rely on cutaneous gas exchange as well as cardiovascular circulation for respiration. In addition, fish are known to change stroke volume rather than heart rate to meet metabolic demands (Stevens and Randall, 1967; Rombough and Ure, 2017). Despite our results, there is strong evidence that GPER has an important metabolic role through control of insulin secretion and glucose tolerance in mammalian models (Haas *et al.*, 2009; Davis *et al.*, 2014). In fish, insulin is known to be more responsive to amino acids than glucose. This presents a rich area for future research from an aquatic toxicological perspective.

Mixture toxicity has become an increasingly important area of aquatic toxicological research. Due to high variability of wastewater constituents, spatial and temporal factors often limit the applicability of whole effluent toxicity assessments. Using whole effluent toxicity data from a regulatory perspective can thus be challenging if the contribution of toxicity from each compound is not fully understood. Thus, investigating the interaction of multiple toxicants can provide valuable information about wastewater toxicity. A major goal of this thesis was to determine if the co-presence of HPβCD could alter the embryonic toxicity of EE2. We observed that the EE2-induced bradycardia was eliminated by the presence of HPβCD at 120 and 144 hours post fertilization (hpf). HPβCD is a large

hydrophilic compound that has the ability to include EE2. Given GPER is located intracellularly, it was hypothesized that HPβCD was impeding the interaction of EE2 and GPER. Uptake of EE2 across the chorion was not impeded by HPβCD, indicating that HPβCD may be able to cross the chorion pore canals. This presents an area of future research, where dechorionating the embryo following exposure to EE2 and HPβCD, may provide insight regarding the uptake of EE2 into tissues of the developing embryo. HPβCD is a unique compound from an aquatic toxicological perspective; we have demonstrated its' ability to reduce guest toxicity, however in a wastewater treatment plant there is also the possibility that HPβCD will cause the mobilization from sediment of various toxicants. The solubility of compounds that that might otherwise have been adsorbed to sludge may significantly increase in the presence of HPβCD, thereby altering toxicant distribution and bioavailability in the environment.

Overall, this thesis has contributed to the understanding of EE2 toxicity through a non-classical estrogen toxicity pathway. We have demonstrated that embryonic heart rate can be modified by GPER activation and not ERα or ERβ, highlighting a potential biomarker for non-genomic estrogen toxicity. More notably, GPER function could be a missing element in our current understanding of estrogen toxicity within aquatic ecosystems. Results from this thesis have also revealed the ability of HPβCD to alter EE2 toxicity. This thesis has elucidated many avenues of future research that could greatly improve our understanding of estrogen toxicity in aquatic systems.

6. References

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7. Appendices

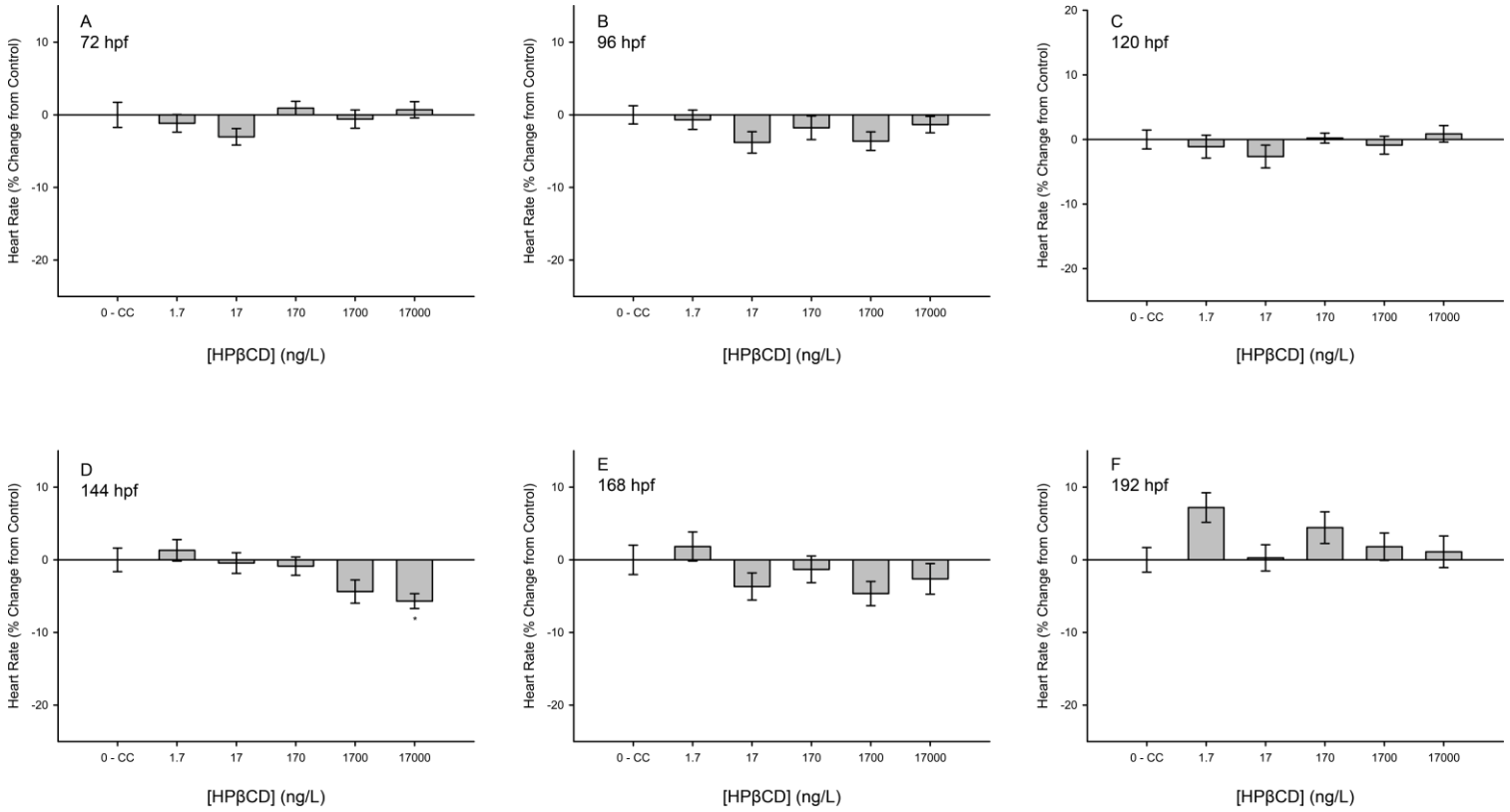

Figure S19: Mean heart rate ± standard error (% change of control) of embryonic Japanese medaka (*O. latipes*) exposed to hydroxypropyl-β-cyclodextrin (HPβCD). The heart rate of each embryo was assessed once every 24 hours at 72 (A), 96 (B), 120 (C): 144 (D), 168 (E), and (192) hours post fertilization (hpf). Significant differences between treatment and the carrier control (CC) (0.01% acetone) are represented by (*) ($P \leq 0.05$) (ANOVA), n=15 embryos per treatment.

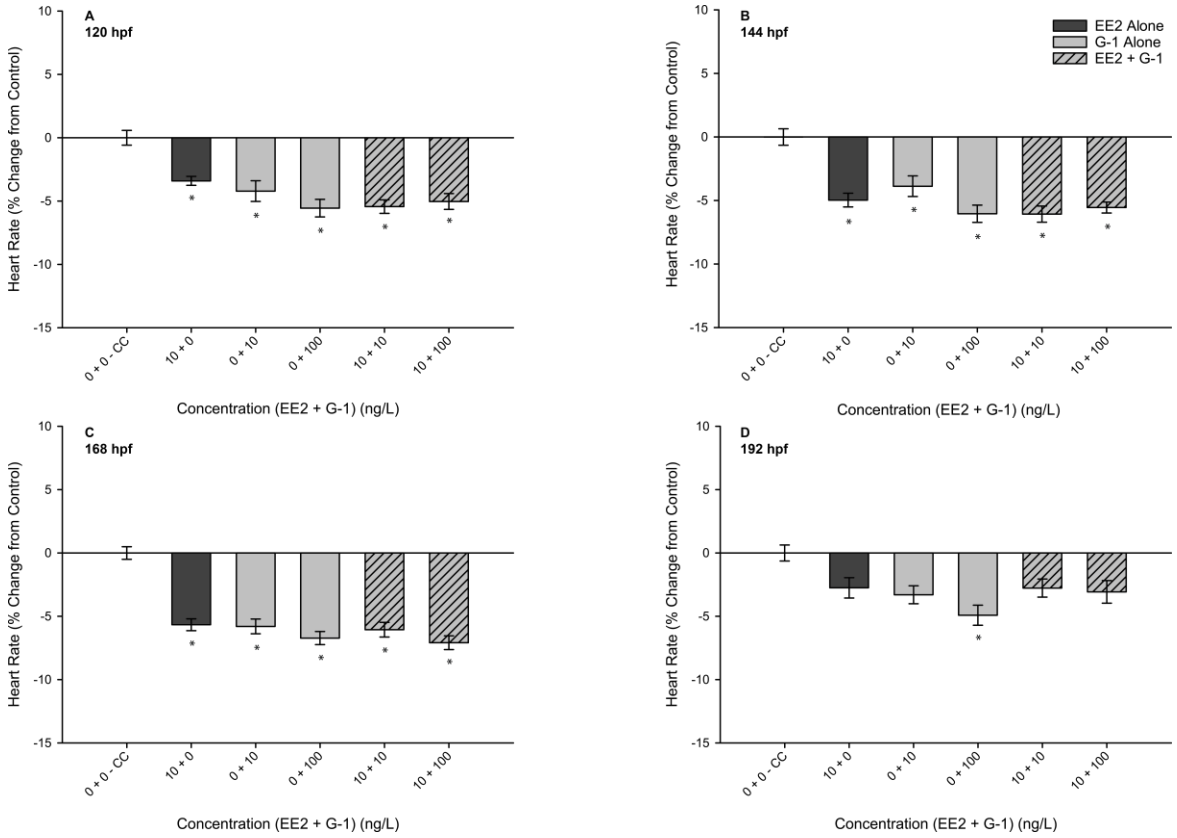

Figure S20: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, G-1, a G Protein-Coupled estrogen receptor (GPER) agonist alone, or EE2 + G-1 at 120 (A), 144 (B), 168(C) and 192 (D) hours post fertilization (hpf). Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA.

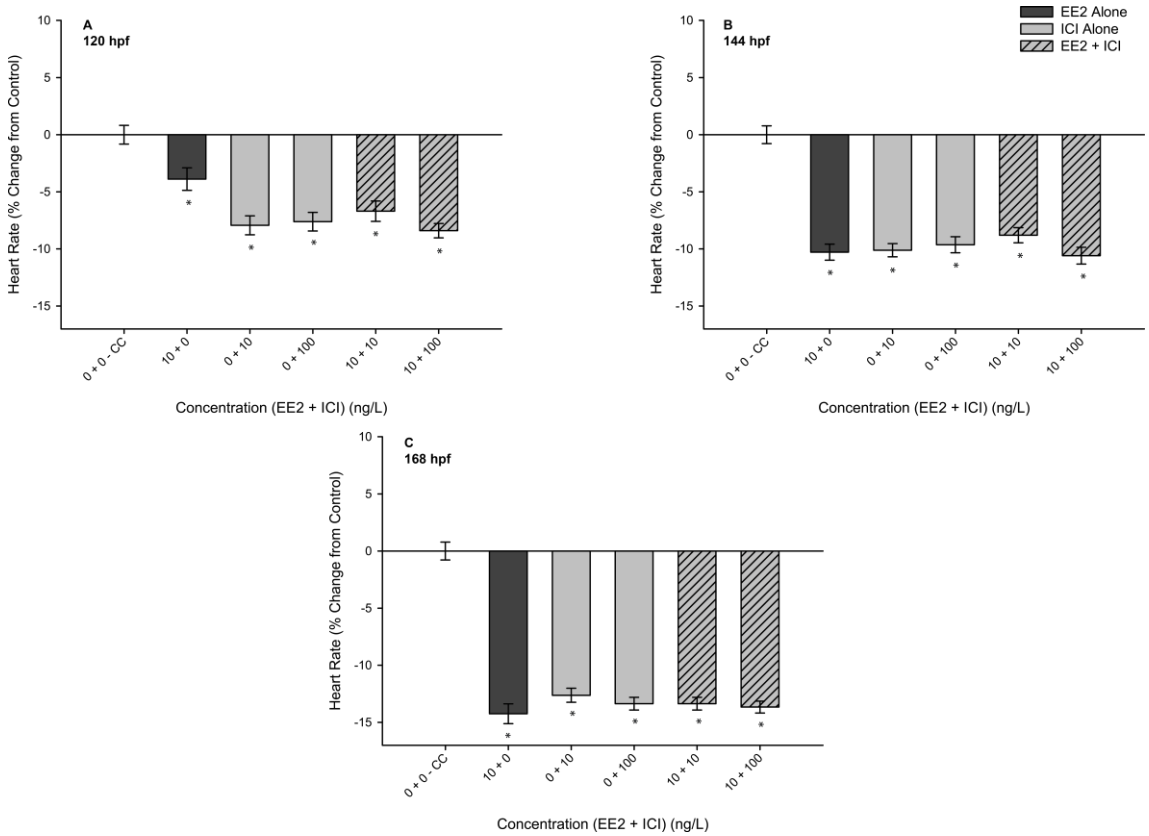

Figure S21: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, Fulvestrant (ICI) (GPER agonist and ERα and ERβ antagonist) alone, or EE2 + ICI at 120 (A), 144 (B), and 168 (C) hours post fertilization (hpf). Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA.

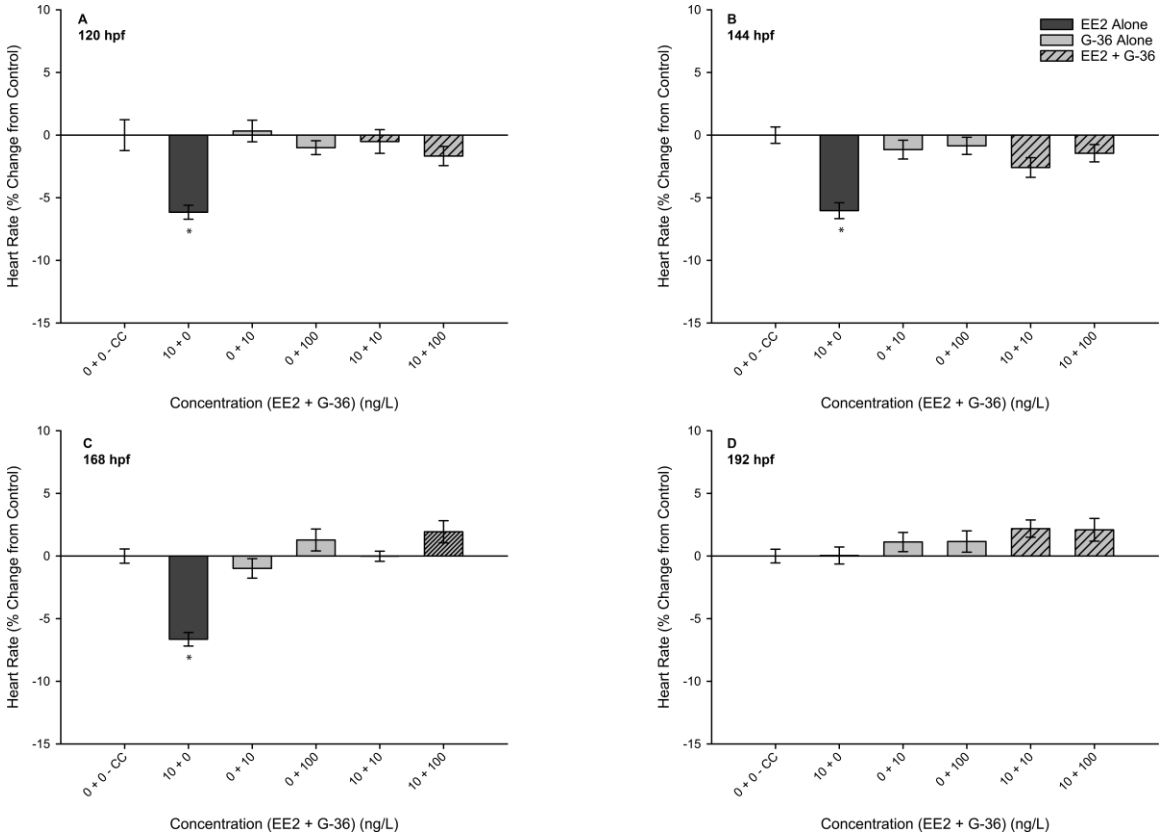

Figure S22: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, G-36 (GPER antagonist) alone, or EE2 + G-36 at 120 (A), 144 (B), 168 (C), 192 (D) hours post fertilization (hpf). Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA.

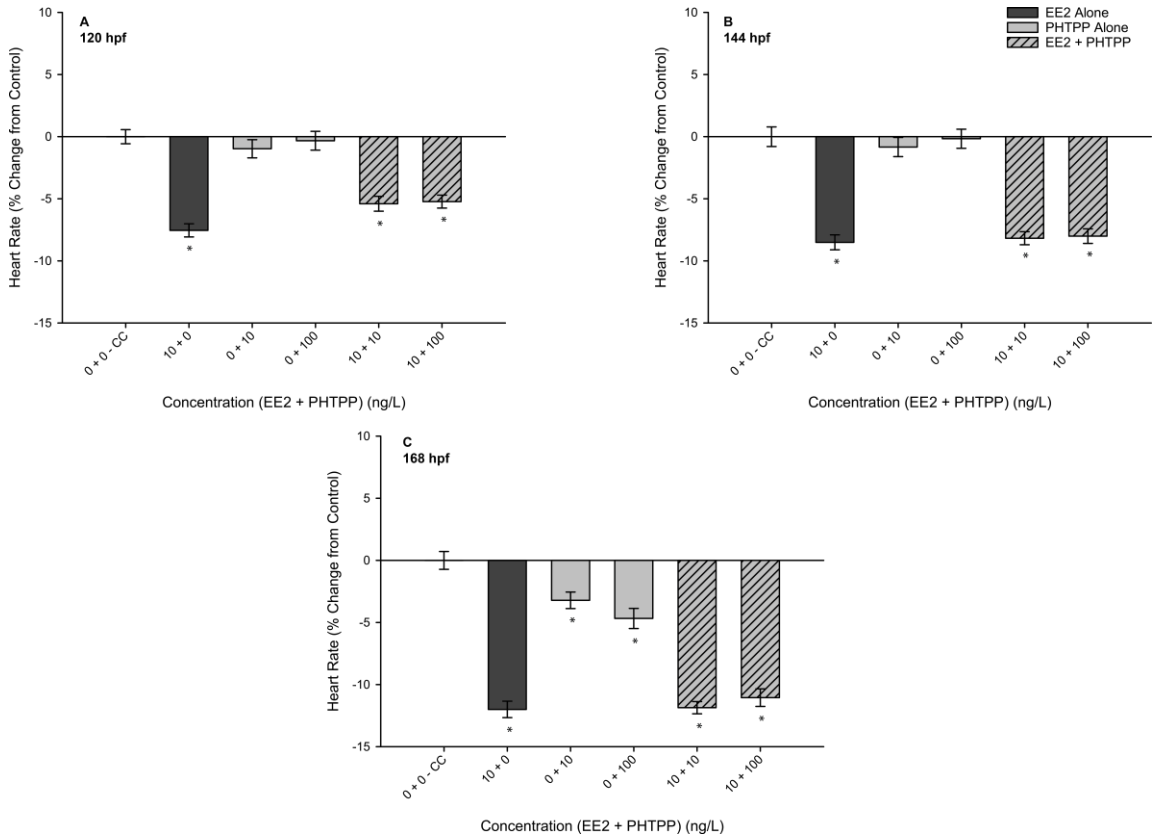

Figure S23: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, PHTPP (ERβ antagonist) alone, or EE2 + PHTPP at 120 (A), 144 (B), 168 (C) hours post fertilization (hpf). Each treatment consists of three replicates containing 7 embryos per replicate. Significant differences from the control are represented by (*) ($P \leq 0.05$), ANOVA.

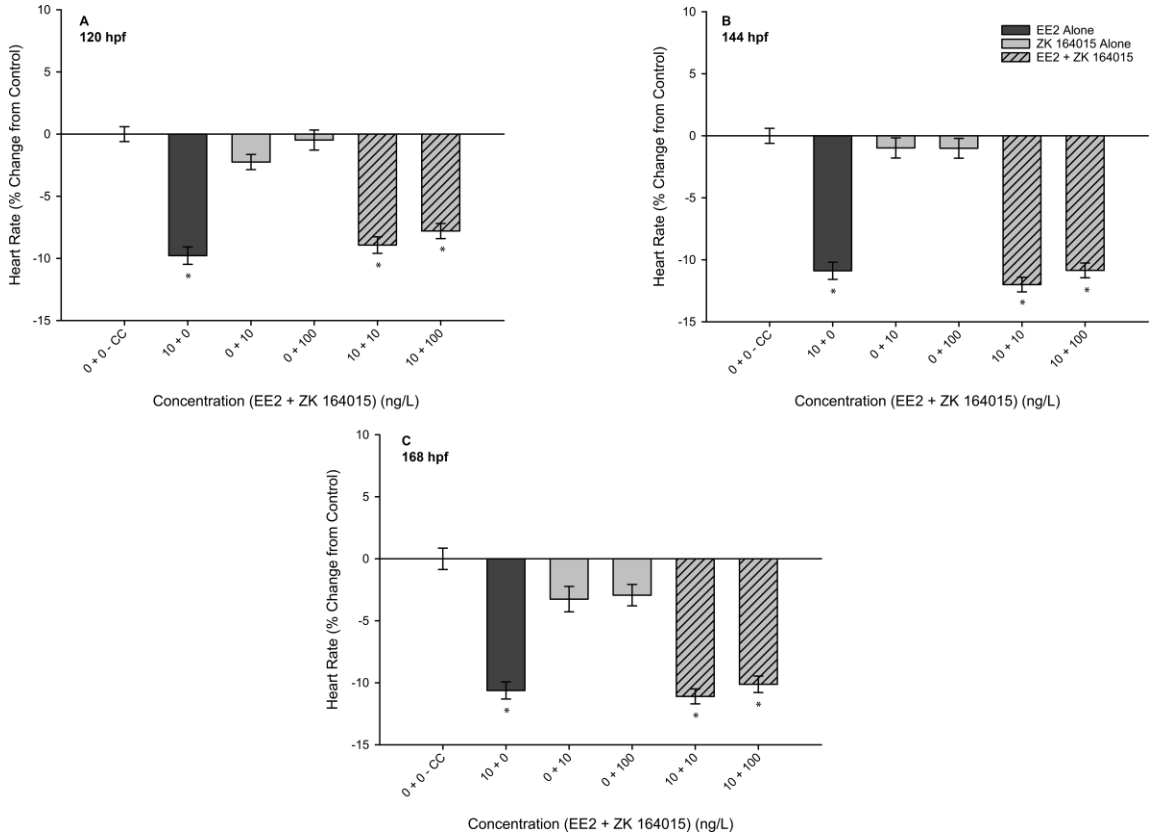

Figure S24: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, ZK 164015 (ERα antagonist) alone, or EE2 + ZK 164015 at 120 (A), 144 (B), and 168 (C). Each treatment consists of three replicates containing 7 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA.

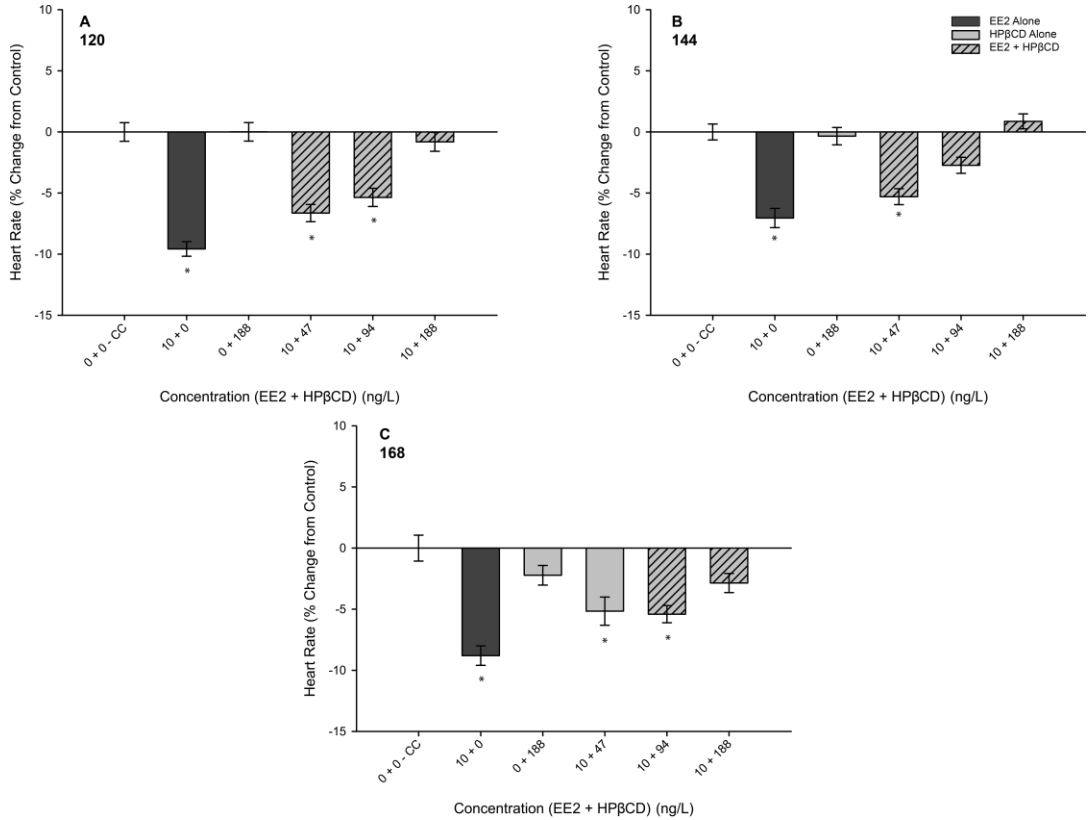

Figure S25: Mean heart rate ± SE (% difference from control) of embryonic Japanese medaka (*O. latipes*) exposed to 17α-ethinylestradiol (EE2) alone, hydroxypropyl-β-cyclodextrin (HPβCD) alone, or EE2 + HPβCD at 120 (A), 144 (B), and 168 (C). Each treatment consists of three replicates containing 7 embryos per replicate. Significant difference from the control is represented by (*) ($P \leq 0.05$), ANOVA.

Table S14: Uptake rangefinder of ^{14}C-17α-ethinylestradiol (^{14}C-EE2) in embryonic Japanese medaka (*O. latipes*). Mass of ^{14}C-EE2 per embryo was determined from one replicate of 40 embryos.

[^{14}C-EE2] (ng/L)	Mass of ^{14}C-EE2 per Embryo (ng)	Number of Replicates
100	112.0	1
1000	415.0	1
10000	665.6	1